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(57) Abstract

This invention provides an isolated nucleic acid which encodes Axin. This invention further provides an isolated nucleic acid which encodes a polypeptide comprising the amino acid sequence of Axin. This invention further provides a purified wildtype or mutant Axin. This invention further provides an oligonucleotide capable of distinguishing nucleic acids encoding mutant or wildtype Axin. This invention also provides various methods of use, such as a method for determining whether a subject carries a mutation in the Axin gene, a method of determining whether a subject has a predisposition for cancer, a method for treating a subject who has a predisposition to cancer, a method for determining whether a subject has cancer, a method for detecting a mutation in cancerous cells of the subject, a method of suppressing cells unable to regulate themselves and a method for identifying a chemical compound which is capable of suppressing cells unable to regulate themselves. This invention also provides a variety of pharmaceutical compositions and a method of treating a subject who has cancer comprising administration of the pharmaceutical compositions. This invention also provides a transgenic, nonhuman mammal, specifically a transgenic expressing mutant Axin.

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AXIN GENE AND USES THEREOF

This application claims priority of U.S. Serial No. 08/890,865, filed July 10, 1997, the contents of which are hereby incorporated by reference into the present application.

The invention disclosed herein was made with Government support under Grant No. DK-46934 from the National Institutes of Health of the United States Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various publications are referenced in parenthesis. Full citations for these publications may be found listed at the end of the specification. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein.

Introduction

A fundamental problem in mammalian embryology is the mechanism by which the egg cylinder, an epithelial cup in which only the dorsal-ventral axis is established, gives rise to an embryo with anterior-posterior (A-P) polarity. In the mouse, the earliest morphological manifestation of the A-P axis is the delamination of mesoderm in the primitive streak at ~E6.5. The position of the streak cannot be predicted by earlier morphological asymmetries in the embryo (Gardner et al., 1992), and the regulative abilities of early mouse embryos appear to rule out axis determination by localized determinants from the egg. While a few secreted factors or transcription factors are expressed asymmetrically in the egg cylinder shortly before primitive streak formation, and thus might be involved in induction of the streak, their roles in this process have not been

established (reviewed by Bachvarova, 1996; Conlon and Beddington, 1995).

In the amphibian embryo, the dorsal-ventral axis (the second axis to be specified, analogous to the A-P axis of the mouse) is determined by the point of sperm entry and subsequent cortical rotation. This rotation generates the Nieuwkoop Center, a group of dorsal/vegetal blastomeres that induce formation of the Spemann organizer. Transplantation of the Nieuwkoop Center or the organizer to an ectopic position induces the formation of a complete secondary axis, i.e., notochord, somites, neural tube, and head structures (reviewed by Slack, 1994). Recent studies suggest that the formation of the Nieuwkoop Center depends on activation of components of the Wnt signaling pathway (Carnac et al., 1996; Fagotto et al., 1997; Wylie et al., 1996). The Wnts are a family of secreted polypeptides related to Drosophila wingless, whose receptors are believed to be members of the frizzled family (reviewed by Miller and Moon, 1996). The next known component of the signaling pathway is Dishevelled (Dsh), a cytoplasmic protein that, when activated by a Wnt signal, inhibits the activity of glycogen synthase kinase-3 (GSK-3). In the absence of a Wnt signal, GSK-3 activity leads (directly or indirectly) to the phosphorylation and consequent degradation of β-catenin. In the presence of a Wnt signal, GSK-3 is inhibited, increasing the cytosolic level of β-catenin, and promoting its interaction with downstream effectors.

A role for the Wnt signaling pathway in development of the amphibian embryonic axis was revealed by the ability of several Wnts, or downstream factors, to induce an ectopic axis when injected into Xenopus embryos (Miller and Moon, 1996). Furthermore, components of this pathway are required for normal axial development because depletion of maternal β-catenin mRNA (Heasman et al., 1994), or sequestration of β-catenin to the plasma membrane (Fagotto et al., 1996), results in ventralized embryos that fail to develop a dorsal

axis. However, it is not clear whether a Wnt ligand triggers Nieuwkoop Center formation, or whether downstream components of the Wnt pathway are activated by some other mechanism (Hoppler et al., 1996; Miller and Moon, 1996; Sokol, 1996). The Nieuwkoop Center is thought to induce a Spemann organizer by secreting a (yet to be identified) diffusible signal (Fagotto et al., 1997; Wylie et al., 1996), which may act synergistically with mesoderm-inducing factors, such as Activin and Vg1, to activate the expression of dorsal-specific genes, such as Goosecoid (Watabe et al., 1995). Dorsoventral patterning of the mesoderm is further controlled by opposing signals emanating from the organizer and the ventral mesoderm: a ventral bone morphogenetic protein (BMP) signal represses dorsal genes, while in the dorsal side the secreted factors Noggin, Chordin and Follistatin directly inhibit BMPs (Hogan, 1996).

While little is known about the molecular control of axis formation in mammalian embryos, a potential source of insight is the study of mouse mutants that affect this process (Conlon and Beddington, 1995; St-Jacques and McMahon, 1996), such as *Fused* (*Fu*). Two spontaneous alleles of *Fu*, called *Kinky* (Fu^{Ki}) and *Knobbly* (Fu^{Kb}), and a transgenic insertional allele, Fu^{Tg1} (previously called *Hε46*), carry recessive mutations that are lethal at E8-E10 (Gluecksohn-Schoenheimer, 1949; Jacobs-Cohen et al., 1984; Perry et al., 1995). In addition to neuroectodermal and cardiac abnormalities, a remarkable property of many early post-implantation embryos homozygous for these three mutant alleles is a duplication of the embryonic axis. This phenotype, unique among mouse mutants, led nearly 40 years ago to the suggestion that *Fu* may play a role in the specification of the embryonic axis (Gluecksohn-Schoenheimer, 1949). *Kinky*, *Knobbly* and a third spontaneous allele, *Fused* (Fu^{Fu}), but not Fu^{Tg1}, also have dominant effects that include transient bifurcations of the fetal tailbud, asymmetric fusion of vertebrae leading to tail kinks, deafness, and neurological defects (Lyon et al.,

1996).

The cloning of this locus with the aid of a transgene insertion was previously described (Perry et al., 1995). Here, the isolation and sequence of cDNA clones, and the genomic structure of the wild type (WT) and Fu^{Tg1} alleles are reported. Analysis of the Fu^{Fu} and Fu^{Kb} alleles (Vasicek et al., manuscript submitted) has shown that both are caused by retroviral insertions. Because two mutant alleles causing axial duplications in homozygous embryos, Fu^{Tg1} and Fu^{Kb}, disrupt production of the major mRNA, the normal gene product may *negatively* regulate a critical step in the formation of the embryonic axis. This hypothesis is supported by studies in Xenopus embryos, which demonstrate that dorsal injection of WT *Fused* mRNA blocks axis formation, while ventral injection of a dominant-negative mutant form induces an ectopic axis. Co-injection with factors acting at various steps in axis formation reveals that Fused exerts its effects at a very early stage, by specifically inhibiting signal transduction through the Wnt pathway in the Nieuwkoop Center. Thus, analysis of the *Fu* locus has identified a novel inhibitor of the Wnt signaling pathway, and suggests that the same pathway regulates an early step in embryonic axis formation in mammals and amphibians. To avoid confusion with the unrelated Drosophila gene *fused*, applicants have renamed the *Fu* gene "*Axin*", for "axis inhibition".

SUMMARY OF THE INVENTION

This invention provides an isolated nucleic acid which encodes Axin.

This invention also provides an isolated nucleic acid which encodes a polypeptide comprising the amino acid sequence of Axin.

This invention also provides a polypeptide comprising the amino acid sequence of Axin. This invention also provides a purified wildtype Axin or purified mutant Axin.

This invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleotides present within a nucleic acid which encodes wildtype Axin without hybridizing to a nucleic acid which encodes mutant Axin, and an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleotides present within the nucleic acid which encodes mutant Axin without hybridizing to a nucleic acid which encodes wildtype Axin.

This invention also provides a method for determining whether a subject carries a mutation in the axin gene which comprises (a) obtaining an appropriate nucleic acid sample from the subject and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject carries a mutation in the axin gene.

This invention further provides a method for determining whether a subject has a predisposition for cancer which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has a predisposition for cancer.

This invention further provides a method for treating a subject who has a predisposition to cancer by either introducing the isolated nucleic acid encoding the wildtype Axin or an effective amount of the wildtype human homolog of Axin and a pharmaceutically acceptable carrier, so as to thereby treat the subject who is susceptible to cancer.

This invention also provides a method for determining whether a subject has cancer, which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has cancer.

This invention also provides a method for detecting a mutation in cancerous cells of the subject which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby detect a mutation in the cancerous cells of the subject.

This invention further provides a method of suppressing cells unable to regulate themselves by either introducing the isolated nucleic acid encoding wildtype Axin or wildtype Axin in an amount effective enough to suppress the cells.

This invention further provides a method of treating a subject who has cancer by either introducing the isolated nucleic acid encoding wildtype Axin or the protein itself in an effective amount.

This invention further provides a method for identifying a chemical compound which is capable of suppressing cells unable to regulate themselves in a subject which comprises (a) contacting mutant Axin with the chemical compound under conditions permitting binding between the mutant Axin and

the chemical compound; (b) detecting specific binding of the chemical compound to the mutant Axin; and (c) determining whether the chemical compound inhibits the mutant Axin so as to identify a chemical compound which is capable of suppressing cells unable to regulate themselves.

This invention further provides pharmaceutical composition comprising a chemical compound capable of inhibiting cancer, an antisense molecule capable of inhibiting an isolated nucleic acid encoding mutant Axin, or purified Axin in an amount effective to treat cancer and a pharmaceutically effective carrier.

This invention further provides a method of treating a subject who has cancer comprising administration of an effective amount of the above-identified pharmaceutical composition

This invention further provides transgenic, nonhuman mammal comprising an isolated nucleic acid encoding Axin, specifically the mutant protein.

BRIEF DESCRIPTION OF THE FIGURES

Abbreviations: The amino acid residues are abbreviated as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. CON: consensus sequence.

Figure 1. Structure of the WT *Axin* gene and the transgenic allele *Axin*^{Tg1}. In *Axin*^{Tg1}, a random transgene insertion (Perry et al., 1995) was accompanied by a deletion including exon 2. Exon 1 is located in a CpG island, as indicated by the frequency of CpG or GpC dinucleotides per 100 bp, and the ratio of CpG/GpC, calculated at 50 bp intervals (left inset). A genomic probe used to isolate cDNA clones is indicated below the inset. The open box in exon 10 represents the 3' UTR. The inset at right shows the origin of form 1and 2 alternatively spliced mRNAs. The cDNA sequences corresponding to exon 1 are 1-308; exon 2, 309-1267; exon 3, 1268-1408; exon 4, 1409-1505; exon 5, 1506-1643; exon 6, 1644-2161; exon 7, 2162-2332; exon 8, 2333-2578; exon 8A, 2579-2686; exon 9, 2687-2854; and exon 10, 2855-3731. The RGS region is encoded in exon 2, and the Dsh homology in exons 9-10. Restriction sites: N, NotI; S, SacII; X, XbaI; B, BamHI.

Figures 2A-2C. Expression of *Axin* mRNA in adult tissues, embryos and ES cells.

2A. Northern blot. ES cells are WT (+/+) or *Axin*$^{Tg1/Tg1}$ (-/-). Sg, salivary gland; Th, thymus; Te, testis; Lu, lung; He, heart; Ki , kidney; Br, brain; Ov, ovary; Sp, spleen; Li, liver.

2B. Whole mount *in situ* hybridization analysis of *Axin* mRNA in (left to right) two E7.5 , two E8.5 and one E9.5 WT embryos. Scale bar, 0.2mm.

2C. Expression of both mRNA isoforms in tissues and ES cells, detected by RT-PCR using primers flanking the 108 bp sequence encoded by exon 8A. The upper band (563

bp) represents form 2 mRNA and the lower band (455 bp) form 1. C, control with no added cDNA. m, 123 bp ladder.

Figures 3A-3D. Amino acid sequence of mouse Axin and its human and chicken homologs, and similarity to RGS and Dsh proteins.

3A-3B. Mouse (m), human (h) and chick (c) Axin sequences. Identical residues are highlighted in black, blue or red and conserved residues in gray. RGS and Dsh homologies are highlighted in blue and red, respectively. The mouse sequence begins with the first residue encoded by the cDNA sequence, and the first two Met residues are indicated by *. Also indicated is a 36 aa segment included in murine and human form 2. The mouse and human form 1 and the chicken sequence contain a bipartite NLS consensus at position 749 (K/R, K/R, 10 aa spacer, followed by 3 K/R in the next 5 residues), which is interrupted in form 2. In addition, mAxin includes a second NLS at aa 59. The murine sequence contains one consensus site for tyrosine phosphorylation (aa 192-199), and several for cAMP- and cGMP-dependent protein kinase, protein kinase C, casein kinase II, and GSK-3. The site of intron 6, where *Axin*^{Fu} contains a proviral insertion, is marked by a green triangle, and the site where exon 7 is interrupted in *Axin*^{Kb}, by a magenta triangle.

3C. Alignment of the RGS domains of Axin and 8 human or rat RGS proteins.

3D. Alignment of a 51 aa segment of Axin with a similar region in Drosophila Dsh and two murine homologs.

Figures 4A-4C. Dorsal injection of *Axin* mRNA ventralizes Xenopus embryos.

4A. Ventralization by dorsal injection of *Axin*, and rescue

by *β-catenin* or *Siamois* but not *Xwnt8*. 2 ng of *Axin* mRNA, either alone or together with the other mRNA indicated, was injected into each of two dorsal blastomeres at the 4-cell stage. Embryos were evaluated at the tadpole stage (Table 1), and examples are shown. The amount of *Xwnt8* (20 pg), *β-catenin* (300 pg) or *Siamois* (100 pg) mRNA used was the minimal amount required to obtain full axis induction when each was injected alone in one ventral blastomere (see Figs. 5A & 5B). Scale bar, 1mm.

4B. Dorsal injection of *Axin* reduces expression of dorsal markers *Siamois*, *Goosecoid*, *Chordin*, and *Xnr3*, but not the ubiquitously expressed elongation factor *EF1*. Each column shows the RT-PCR analysis of a pool of uninjected embryos or embryos injected at the 4-cell stage with *Axin* or control *β-gal* mRNA (2 ng), and grown to early gastrulae. -RT, control experiments in which RNA from uninjected embryos was processed without reverse transcriptase.

4C. Dorsal co-injection of *β-catenin* with *Axin* restores expression of *Siamois* and *Goosecoid*, and co-injection of *Siamois* restores *Goosecoid* expression, while co-injection of *Xwnt8* has no effect. Note that the injected *Siamois* (not detected with the primers used in this assay) does not induce expression of endogenous *Siamois*.

Figures 5A-5C. Ability of *Axin* to block ectopic axis formation.

5A. Ventral co-injection of *Axin* mRNA inhibits ectopic axis formation by upstream components of the Wnt pathway (Xwnt8, Xdsh and dnGSK-3), but not by *β-catenin* or *Siamois*, nor by *Activin*, *Noggin* or *ΔBMPR*. mRNA encoding the indicated dorsalizing factor was injected subequatorially in one ventral blastomere at the 4-8 cell stage, with or without 1 ng *Axin*, and embryos were examined for axial duplications at the late neurula - tailbud stage. The fraction of embryos with duplicated

axes is indicated above each bar. mRNAs were injected in the minimal amounts needed to induce ectopic axes at high frequency: 10-20 pg *Xwnt8*, 1.5 ng *Xdsh*, 2 ng *dnGSK-3*, 300 pg *β-catenin*, 100 pg *Siamois*, 7.5 pg *Activin*, 200 pg *noggin*, or 1 ng *ΔBMPR* (Fagotto et al., 1997). *Activin*-induced secondary axes were generally very incomplete. Higher amounts of *Activin* mRNA lead to uninterpretable phenotypes.

5B. Examples of injected embryos. Scale bar, 2mm.

5C. Co-injection of *Axin* mRNA inhibits induction of the dorsal marker *Goosecoid* by *Xwnt8*, but not by *Activin*, *Noggin*, or *ΔBMPR*. Ectopic expression of *Goosecoid* in the ventral half of early gastrulae (stage 10 1/2) was analyzed by RT-PCR. Dorsal (D) and ventral (V) halves of uninjected embryos served as positive and negative controls (ctrl) for normal expression of *Goosecoid*.

Figures 6A-6G. Axis duplications in Xenopus embryos injected ventrally with *ΔRGS*, and in mouse embryos homozygous for the loss-of-function *Axin^{Tg1}* allele.

6A & 6B. Xenopus embryos with axis duplications caused by injection of 2 ng *ΔRGS* in one ventral blastomere (*6A*) or 1 ng *ΔRGS* in two ventral blastomeres (*6B*). The embryo in *6B* is also strongly dorsalized. Scale bars, 0.5mm.

6C. Frequency of axis duplications in embryos injected with *Axin*, *ΔRGS*, or *ΔRGS* together with *Axin* or *C-cadherin*.

6D. Ectopic expression of dorsal markers in embryos injected ventrally with *ΔRGS*. Each column shows the RT-PCR analysis of the dorsal (D) or ventral (V) halves of a pool of embryos. In uninjected embryos, *Siamois*, *Goosecoid*, *Chordin*, and *Xnr3* are expressed dorsally. Ventral injection of *ΔRGS*, but not *Axin*, induces ectopic expression of the four dorsal markers.

6E-6G. Lateral view of a normal E7.5 mouse embryo (e6E) and two E8.5 *Axin$^{Tg1/Tg1}$* embryos with axial

duplications (6F and 6G), visualized by *in situ* hybridization to *HNF-3β*, a marker of anterior axial mesoderm (Sasaki and Hogan, 1994). White arrows, primary axes; black arrowheads, ectopic axes. Scale bars, 0.2mm.

Figure 7. Model for the inhibitory effect of Axin on Wnt signal transduction. Established components of the Wnt pathway in the Nieuwkoop Center are indicated by blue symbols and solid black arrows, and positions where Axin might inhibit the pathway are indicated by red symbols and dashed arrows. GSK-3 promotes the degradation of β-catenin, while Wnt signals inhibit GSK-3 (via Dsh) and lead to accumulation of cytosolic β-catenin and expression of Siamois. Axin blocks the stimulation of this pathway by Wnt, Dsh or dominant-negative GSK-3, but not by overexpression of β-catenin or Siamois. Three alternative hypotheses are illustrated : (1) Axin might inhibit a protein phosphatase (PP2A) that may otherwise dephosphorylate substrates of GSK-3; (2) Axin might stimulate the activity of GSK-3 through an unknown mechanism; (3) Axin might inhibit, via its RGS domain, the transmission of a second signal (signal 2) involving a G-protein-coupled receptor, which would otherwise stimulate the Wnt pathway downstream of GSK-3. See text for further details.

Figure 8. Amino acid sequence of murine Axin (Sequence I.D. No. 1).

Figures 9A-9B. Nucleic acid sequence of murine Axin (Sequence I.D. No. 2).

Figures 10A-10B. Nucleic acid sequence of human Axin (Sequence I.D. No. 3).

Figure 11. Amino acid sequence of human Axin. (Sequence I.D. No. 4).

DETAILED DESCRIPTION

Throughout this application, references to specific nucleotides are to nucleotides present on the coding strand of the nucleic acid. The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

C=cytosine	A=adenosine
T=thymidine	G=guanosine

A "gene" means a nucleic acid molecule, the sequence of which includes all the information required for the normal regulated production of a particular protein, including the structural coding sequence, promoters and enhancers.

The nucleic acids or oligonucleotides of the subject invention also include nucleic acids or oligonucleotides coding for polypeptide analogs, fragments or derivatives which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These nucleic acids or oligonucleotides include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The nucleic acids and oligonucleotides described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as

products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

This invention provides for an isolated nucleic acid which encodes Axin. This isolated nucleic acid may be DNA or RNA, specifically cDNA or genomic DNA. This isolated nucleic acid also encodes mutant Axin or the wildtype protein.

Specifically, the isolated nucleic acid encodes a mouse wildtype Axin. This isolated nucleic acid may have the sequence designated Seq. I.D. No.: 2. Also, the isolated nucleic acid may have substantially the same amino acid sequence as the sequence desiganted Seq. I.D. No.: 1.

The isolated nucleic acid may also encode a human Axing having substantially the same amino acid sequence as the sequence designated Seq. I.D. No.: 4. Specifically the isolated nucleic acid has the sequence designated Seq. I.D. No.: 3.

This isolated nucleic acid may also encode a polypeptide comprising the amino acid sequence of Axin.

As used in this application, "Axin" means and includes any polypeptide having Axin activity, e.g. negative regulation of the Wnt signalling pathway, and having an amino acid sequence homologous to the amino acid sequence of mouse Axin (the sequence of which is set forth in Sequence I.D. No.: 1). Thus, this term includes any such polypeptide whether naturally occurring and obtained by purification from natural sources or non-naturally occuring and obtained synthetically, e.g. by recombinant DNA procedures. Moreover, the term includes any such polypeptide whether its

sequence is substantially the same as, or identical to the sequence of any mammalian homolog of the human polypeptide, e.g. murine, bovine, porcine, etc. homologs. Additionally, the term includes mutants or other variants of any of the foregoining which retain at least some of the enzymatic activity of nonmutants or nonvariants.

The invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from Axin, but which do not produce phenotypic changes.

However, a mutant Axin will not exhibit the same phenotype as the wildtype Axin. For example, a cell containing a mutant version of the axin gene will express a protein unable to negatively regulate the Wnt signalling pathway.

The nucleic acid of the subject invention also include nucleic acids that encode for polypeptide analogs, fragments or derivatives which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (including deletion analogs containin less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of the naturally-occuring forms.

The polypeptide of the subject invention also includes analogs, fragments or derivatives which differ from naturally-occurring forms, but Axin activity.

This invention also provides a vector comprising an isolated nucleic acid encoding Axin. The isolated nucleic acid of the vectors is operatively linked to a promoter of RNA transcription which maybe, or is identical to, a bacterial, yeast, insect or mammalian promoter. The vector may be a plasmid, cosmid, yeast artificial chromosome (YAC),

bacteriophage or eukaryotic viral DNA. Specifically, this invention provides two cosmids designated Genbank Accession No. Z69667 and Z81450, respectively.

Further other numerous vector backbones known in the art as useful for expressing proteins may be employed. Such vectors include but are not limited to: adenovirus, simian virus 40 (SV40), cytomegalovirus (CMV), mouse mammary tumor virus (MMTV), Moloney murine leukemia virus, murine sarcoma virus, and Rous sarcoma virus, DNA delivery systems, i.e liposomes, and expression plasmid delivery systems.

This invention also provides a vector system for the production of a polypeptide which comprises the vector in a suitable host. Suitable host includes a cell which includes, but is not limited, prokaryotic or eukaryotic cells, e.g. bacterial cells (including gram positive cells), yeast cells, fungal cells, insect cells and animal cells.

Suitable animal cells include, but are not limited to, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells. Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH 3T3, CHO cells, Ltk$^-$ cells, etc. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, electroporation.

This invention also provides a method for producing a polypeptide (e.g. Axin) which comprises growing a host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced. Methods of recovering polypeptides produced in such host vector systems are well-known in the art and typically include steps involving cell lysis, solubilization and chromatography.

This invention also providies a method of obtaining a

polypeptide in purified form which comprises: (a) introducing a vector, as described above, into a suitable host cell; (b) culturing the resulting cell so as to produce the polypeptide; (c) recovering the polypeptide produced in step (b); and (d) purifying the polypeptide so recovered. As discussed above the vector may include a plasmid, cosmid, yeast artificial chromosome, bacteriophage or eukaryotic viral DNA. Also, the host cell may be a bacterial cell (including gram positive cells), yeast cell, fungal cell, insect cell or animal cell. Suitable animals cells include, but are not limited to HeLa cells, Cos Cells, CV1 cells and various primary mammalian cells. Culturing methods useful for permitting transformed or transfected host cells to produce polypeptides are well known in the art as are the methods for recovering polypeptides from such cells and for purifying them.

Using the aforementioned method, this invention also provides a purified wildtype Axin and a purified mutant Axin. Further, this invention also provides a polypeptide comprising the amino acid sequence of Axin, including, but limited to, fusion proteins having part of their amino acid sequence the amino acid sequence of Axin.

This invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encodes a wildtype Axin without hybridizing to a nucleic acid which encodes a mutant Axin. Further, this invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within the nucleic acid which encodes a mutant Axin without hybridizing to a nucleic acid which encodes a wildtype Axin. These oligonucleotides may be DNA or RNA. Such oligonucleotides may be used in accordance with well known standard methods for known purposes, for example, to detect the presence in asample of DNA which will hybridize thereto.

As used herein, "capable of specifically hybridizing" means wherein the oligonucleotide will selectively bind to only sequences which are unique to either nucleic acids encoding wildtype or mutant Axin.

The oligonucleotides include, but are not limited to, oligonucleotides that hybridize to mRNA encoding Axin so as to prevent translation of the protein.

This invention also provides a nucleic acid having a sequence complementary to the sequence of the isolated nucleic acid which encodes Axin. Furthermore, this application also provides an antisense molecule of capable of specifically hybridizing with the isolated nucleic acid encoding mutant Axin.

This invention provides a method for determining whether a subject carries a mutation in the axin gene which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject carries a mutation in the axin gene.

In a specific example of the above-described method, the nucleic acid sample in step (a) is mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises (i) contacting the mRNA with the oligonucleotide capable of detecting only nucleic acid corresponding to mutant Axin under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

As used herein "corresponding to mutant Axin" means capable of specifically hybridizing with a unique sequence of

nucleotides present within the nucleic acid which encodes a mutant Axin without hybridizing to a nucleic acid which encodes a wildtype Axin.

In another specific embodiment, the determining of step (b) comprises (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid encoding Axin with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid, (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

In another embodiment, the determining of step (b) comprises: (i) sequencing the nucleic acid sample of step (a); and (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid encoding wildtype Axin, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

One can also practice the invention, wherein the determining of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

In order to facilitate identification of the nucleic acid from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The detectable marker may be a radioactive isotope, a fluorophor or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (i).

The invention described above may have sample which is blood, tissues or sera.

This invention also provides a method for determining whether a subject has a predisposition for cancer which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has a predisposition for cancer.

In a specific example of the above-described method, the nucleic acid sample in step (a) is mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises (i) contacting the mRNA with the oligonucleotide capable of detecting only nucleic acid corresponding to mutant Axin under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

As used herein "corresponding to mutant Axin" means capable of specifically hybridizing with a unique sequence of nucleotides present within the nucleic acid which encodes a mutant Axin without hybridizing to a nucleic acid which encodes a wildtype Axin.

In another specific embodiment, the determining of step (b) comprises (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid encoding Axin with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid, (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived

from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

In another embodiment, the determining of step (b) comprises: (i) sequencing the nucleic acid sample of step (a); and (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid encoding wildtype Axin, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

One can also practice the invention, wherein the determining of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

In order to facilitate identification of the nucleic acid from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The detectable marker may be a radioactive isotope, a fluorophor or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (i).

This invention also provides where the sample includes, but is not limited to, blood, tissues or sera.

As used herein "cancer" includes, but is not limited to, brain, thyroid, breast, colorectal, gastrointestinal, esophageal carcinomas or melanomas.

This invention also provides a method for treating a subject who has a predisposition to cancer which comprises introducing the isolated nucleic acid encoding wildtype Axin into the subject under conditions such that the nucleic acid expresses wildtype Axin, so as to thereby treat the subject.

This invention also provides a method for treating a subject who has a predisposition to cancer which comprises an effective amount of the wildtype human homolog of Axin and a pharmaceutically acceptable carrier, so as to thereby treat the subject who is susceptible to cancer.

This invention also provides a method for determining whether a subject has cancer, which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has cancer.

In a specific example of the above-described method, the nucleic acid sample in step (a) is mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises (i) contacting the mRNA with the oligonucleotide capable of detecting only nucleic acid corresponding to mutant Axin under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

In another specific embodiment, the determining of step (b) comprises (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid encoding Axin with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid, (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

In another embodiment, the determining of step (b) comprises: (i) sequencing the nucleic acid sample of step (a); and (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid encoding wildtype Axin, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

One can also practice the invention, wherein the determining of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

In order to facilitate identification of the nucleic acid from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The detectable marker may be a radioactive isotope, a fluorophor or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (i).

This invention also provides a method for detecting a mutation in cancerous cells of the subject which comprises: (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby detect a mutation in the cancerous cells of the subject.

In a specific example of the above-described method, the nucleic acid sample in step (a) is mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises (i) contacting the mRNA with the oligonucleotide capable of detecting only nucleic acid corresponding to mutant Axin under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to

thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

In another specific embodiment, the determining of step (b) comprises (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid encoding Axin with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid, (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

In another embodiment, the determining of step (b) comprises: (i) sequencing the nucleic acid sample of step (a); and (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid encoding wildtype Axin, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

One can also practice the invention, wherein the determining of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

In order to facilitate identification of the nucleic acid from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The detectable marker may be a radioactive isotope, a fluorophor or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (i).

This invention also provides a method of suppressing cells

unable to regulate themselves which comprises introducing the isolated nucleic acid encoding wildtype Axin into the cells.

In a specific example of the above-described method, the nucleic acid sample in step (a) is mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises (i) contacting the mRNA with the oligonucleotide capable of detecting only nucleic acid corresponding to mutant Axin under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

In another specific embodiment, the determining of step (b) comprises (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid encoding Axin with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid, (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

In another embodiment, the determining of step (b) comprises: (i) sequencing the nucleic acid sample of step (a); and (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid encoding wildtype Axin, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

One can also practice the invention, wherein the determining

of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

In order to facilitate identification of the nucleic acid from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The detectable marker may be a radioactive isotope, a fluorophor or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (i).

Also, the cells may be unable to regulate themselves because of a mutation in the axin gene or a mutation in the adenomatous polyposis coli gene.

This invention also provides a method of suppressing cells unable to regulate themselves which comprises introducing wildtype Axin into the cells in an amount effective enough to suppress the cells.

This invention also provides a method of treating a subject who has cancer which comprises introducing the isolated nucleic acid encoding wildtype Axin, into the subject so as to thereby treat the cancer.

One well-known means of introducing the isolated nucleic acid, comprises: (a) recovering cancerous cells from the subject; (b) introducing the isolated nucleic acid encoding the wildtype Axin into the cells; and (c) reintroducing the cells of step (b) into the subject so as to treat the subject who has cancer.

This invention further provides a method for identifying a chemical compound which is capable of suppressing cells unable to regulate themselves in a subject which comprises: (a) contacting mutant Axin with the chemical compound under conditions permitting binding between the

mutant Axin and the chemical compound; (b) detecting specific binding of the chemical compound to the mutant Axin; and (c)determining whether the chemical compound inhibits the mutant Axin so as to identify a chemical compound which is capable of suppressing cells unable to regulate themselves.

This method is applicable when the cells are cancerous. For example, when cancerous cells are derived from the abdominal cavity, brain, breast, skin, colon, rectum, esophagus, stomach, thyroid or intestine..

This invention provides pharmaceutical compositions comprising the chemical compound identified by the above-described method of in an amount effective to inhibit cancer and a pharmaceutically effective carrier.

Further this invention also includes a pharmaceutical compositions includes an antisense molecule to an isolated nucleic acid which encodes mutant Axin in an amount effective to treat cancer and a pharmaceutically effective carrier.

This invention also includes a pharmaceutical composition comprising the purified wildtype Axin in an amount effective to treat cancer and a pharmaceutically effective carrier.

One can treat a subject who has cancer by administrating an effective amount of the above-described pharmaceutical composition to the subject who has cancer.

One can administer the above-described compositions by topical, oral, aerosol, subcutaneous administration, infusion, intralesional, intramuscular, intraperitoneal, intratumoral, intratracheal, intravenous injection, or liposome-mediate delivery.

Methods of administration of pharmaceutical compositions are

well-known in the art.

This invention also includes transgenic, nonhuman mammal containing the isolated nucleic encoding Axin, specifically, an isolated nucleic acid encoding mutant Axin.

This invention further provides a method for treating a subject who has a predisposition to cancer which comprises introducing the isolated nucleic acid which encodes Axin, wherein the Axin is wildtype Axin, into the subject under conditions such that the nucleic acid expresses wildtype Axin, wherein the nucleic acid is overexpressed, so as to thereby treat the subject.

This invention provides a method for treating a subject who has cancer which comprises introducing the isolated nucleic acid which encodes Axin, wherein the Axin is wildtype Axin, into the subject under conditions such that the nucleic acid expresses wildtype Axin, wherein the nucleic acid is overexpressed, so as to thereby treat the subject.

In an embodiment of either of the above-described methods the overexpression of the wildtype Axin may be accomplished by putting the introduced nucleic acid under a strong promoter. Methods of inducing overexpression are well known to one of ordinary skill in the art.

This invention provides a method of inhibiting tumorigenesis in a subject which comprises administering an effective amount of a molecule which induces degradation of β-catenin thereby inhibiting tumorigensis. This method may also be used to inhibit cellular transformation, i.e. oncogenic transformation of a normal cell to a cancer cell. In a preferred embodiment of the above-described methods the molecule which induces degradation of β-catenin is Axin or a functionally active mutant of Axin. Alternatively, drugs

or small molecules which act with Axin, i.e. in synergy with Axin to induce degradation of β-catenin may also be used in another embodiment of this method, for example drugs which inhibit enzymes.

This invention provides a method of treating a subject with cancer which comprises administering an effective amount of a pharmaceutical composition comprising a molecule which induces degradation of β-catenin and a pharmaceutically acceptable carrier, thereby treating the subject with cancer. In a preferred embodiment of the above-described method the molecule which induces degradation of β-catenin is Axin or a functionally active mutant of Axin. Drugs or small molecules which act with Axin, i.e. in synergy with Axin to induce degradation of β-catenin may also be used in other embodiments of this method, for example drugs which inhibit enzymes.

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

FIRST SECTION

Experimental Procedures

1. Isolation and characterization of mouse *Axin* cDNA and genomic clones

Primers for *mAxin* are named for the position of their 5' terminus in the cDNA (+ numbers) or in upstream genomic DNA (- numbers). F indicates a forward and R a reverse primer. Sequences are listed 5' to 3'. A 315 bp NotI-StuI genomic probe at the left of the transgene insert (Perry et al., 1995) was used to screen an E8.5 mouse embryo cDNA library, yielding one clone (N7) that was partially colinear with the probe. Using a fragment of N7, 12 more clones were isolated from a WEHI-3 cDNA library (Stratagene). Additional *Axin* cDNA clones were obtained from various libraries using probes from clone N7, but none extended as far 5' as N7. 5' RACE was performed using kidney cDNA, AP1 primer (Advantage cDNA PCR kit, Clonetech) and mAxin primer +98R (caccagccctctctggaacc). The RACE product extending farthest 5' was colinear with clone N7 and contained 4 more bp at the 5' end. To estimate the ratio of forms 1 and 2 mRNA, total RNA was reverse transcribed using oligo-dT primer, and the cDNA was amplified using +2289F (gagggagagaaggagatcag) (Sequence I.D. No. 5) and +2744R (gtagctccccttcttggttag) (Sequence. I.D. No. 6).

Intron/exon structure was determined by restriction mapping and sequencing of genomic subclones and products of long template PCR using primers in adjacent exons. Previously isolated clones (Perry et al., 1995) included exons 1-3, and clones including exons 6-10 were isolated from a strain 129 library. The remaining region was isolated by long template PCR (Boehringer Mannheim) using primers at different positions in the cDNA.

2. Isolation of human and chick Axin cDNA clones

Database searches revealed ESTs (T07178, R75687, T30966, T32063, T15895 and T72547) representing the 3' region of a

human *Axin* homolog (*hAxin*). Additional clones were isolated by 5' and 3' RACE using human placenta RNA (Clonetech Marathon RACE kit). A stage 12-15 chick embryo cDNA library (a gift of Dr. D. Wilkinson) was screened with a *mAxin* probe and four clones containing the same insert in both orientations were isolated. The 3131 bp *cAxin* cDNA sequence contains a polyadenylation signal near the 3' end, but is shorter than the *cAxin* mRNA (~3.6 kb), even after accounting for a poly(A) tail, and thus may lack part of the 5' UTR.

3. **Sequence analysis**

Database searches were conducted using BLAST, and sequence alignments using ClustalW and BOXSHADE. Other RGS sequences are: Q08116 (hRGS1), P41220 (hRGS2), U27768 (hRGS3), U27768 (rRGS4), D31257 and R35272 (hRGS5), U32328 (hRGS7), H87415 (hRGS10), X91809 (hGAIP).

4. **Northern blot and *in situ* hybridization**

20μg of total RNA from embryos, adult tissues or ES cells was run on a formaldehyde-agarose (1.2%) gel, blotted to Genescreen plus (NEN-Dupont), and hybridized with a ^{32}P-DNA probe containing the entire *mAxin* cDNA sequence, as described (Perry et al., 1995). For *in situ* hybridization (Wilkinson, 1992), an anti-sense probe was produced by T7 transcription of a HindIII-SacI fragment of *mAxin* cDNA (bp 765-1065, within exon 2) in pBluescript. A sense probe did not produce a significant signal. The *HNF-3β* probe was produced from clone c21 (Sasaki and Hogan, 1993).

5. **Constructs for Xenopus injection**

Axin cDNAs were cloned into the XhoI site of pCS2+MT (Rupp et al., 1994). The experiments shown employed vector MTPA2, which includes *Axin* form 2 (bp 37 to 3310) and encodes aa 13 to the normal C-terminus. Translation initiates in the N-terminal Myc tag. Three other *Axin* vectors (MTPA1, MTFU1 and MTFU2) were similarly active at ventralization: MTPA1 was identical to MTPA2 except it was derived from a form 1 cDNA. MTFU1 and MTFU2 were identical to MTPA1 and MTPA2, except

they contained a longer 3' UTR (bp 3311-3731). ΔRGS was derived from MTFU1 by deleting cDNA bp 754 -1053. *Siamois* was cloned in pCS2+MT (Fagotto et al., 1997) and *β-catenin* (C-terminally HA-tagged) in pSP36 (Funayama et al., 1995). Other expression vectors were: *Xwnt8* and *Noggin* (gift of Dr. R. Harland), *Activin* (Dr. D. Melton), *ΔBMPR* (Dr. A. Susuki), *dnGSK-3* (GSK-3-K->R, Dr. D. Kimelman) and *Xdsh* (Dr. U. Rothbacher). GSK-3-K->R is a mutant of Xenopus GSK-3β without kinase activity (Pierce and Kimelman, 1995). ΔBMPR is a truncated BMPR lacking the kinase domain (Suzuki et al., 1994).

6. Xenopus injections and analysis of phenotypes

mRNAs were synthesized and injected as previously described (Fagotto et al., 1996; 1997). For RT-PCR, mRNA was extracted from whole early gastrulae (stage 10 1/2) or dissected dorsal and ventral halves, and specific mRNAs were detected as described (Fagotto et al., 1997). *Siamois* primers were: 5' ttgggagacagacatga (corresponds to part of the 5' UTR, present in the endogenous mRNA but not the injected synthetic *Siamois* mRNA) (Sequence I.D. No. 7) and 3': tcctgttgactgcagact (Sequence I.D. No. 8). Other primers were as described (Fagotto et al., 1997). For immunofluorescence, Myc-tagged Axin was detected in frozen sections of early gastrulae using anti-Myc antibody 9E10.2, as described (Fagotto et al., 1996; Fagotto and Gumbiner, 1994).

Results

1. Identification of the *Axin* gene

A genomic probe from the $Axin^{Tg1}$ transgene insertion locus detected a 3.9 kb RNA in wild type embryonic stem (ES) cells but not in $Axin^{Tg1/Tg1}$ ES cells, representing a strong candidate for the *Axin* mRNA (Perry et al., 1995). To isolate cDNA clones, a probe located within a CpG island upstream from the transgene insertion site (Fig. 1) was used to screen a mouse embryo library. One cDNA clone contained a region identical in sequence with the genomic probe,

confirming that it was encoded at the *Axin* locus, and this clone was used to isolate additional overlapping cDNA clones. The composite cDNA sequence was 3623 bp long, excluding the poly(A) tail. All the cDNA clones were co-linear in their regions of overlap, except for a 108 bp sequence present in a fraction of clones following bp 2579, representing an alternative splicing product (Fig. 1). Transcripts lacking this 108 bp segment were termed "form 1" and those containing it "form 2".

2. Genomic organization of *Axin*

Using *Axin* cDNA probes, a series of overlapping clones was isolated from a WT mouse genomic library, and the locations of exons and introns were determined (see Experimental Procedures). As illustrated in Fig. 1, 10 exons were identified, spanning ~56 Kb. The extra 108 bp segment in form 2 mRNA results from the use of an alternative 5' splice site following exon 8, and is designated exon 8A. The longest cDNA clones representing the 3.9 kb mRNA appeared to be missing 25-75 nt from the 5' end, based on RNase H and S1 nuclease mapping studies. Difficulties in cloning these 5' terminal sequences may be a consequence of the very high GC content of the CpG island surrounding the apparent promoter region (Fig. 1). Based on Southern blot hybridization to genomic DNA, and the analysis of multiple cDNA and genomic clones, *Axin* appears to be a single copy gene.

In the $Axin^{Tg1}$ allele, exon 2 and parts of the two flanking introns are deleted. Exons 1 and 3 are separated by a ~600 kb transgene insertion (Fig. 1), a disruption that leads to the absence of the major WT 3.9 kb mRNA in homozygotes (Fig. 2). As described elsewhere (Vasicek et al., manuscript submitted), the $Axin^{Fu}$ allele contains an endogenous intracisternal A particle (IAP) provirus within intron 6, while $Axin^{Kb}$ contains a similar IAP element interrupting exon 7. The $Axin^{Ki}$ allele is apparently extinct.

3. Ubiquitous expression of wild type *Axin* mRNA.

On Northern blots, a major band of ~3.9 kb was observed in all WT adult tissues examined, embryos at E10.5 - E16.5, and ES cells. A 3.0 kb band was also observed at very low levels in some WT tissues and ES cells. In *Axin*$^{Tg1/Tg1}$ ES cells, the 3.9 kb RNA was absent, but a 3.0 kb RNA was observed (Fig. 2A). Because the 3.0 kb mRNA was observed in both WT and *Axin*$^{Tg1/Tg1}$cells, and contains exons 3 - 10 but not 1 and 2, it is likely to be transcribed from a weak promoter downstream from the 3' end of the transgene-induced deletion. Thus, *Axin*^{Tg1} is a loss-of-function allele with respect to the major 3.9 kb mRNA, although it may not be a null allele.

In situ hybridization with WT embryos at E7.5 - E9.5 showed that *Axin* mRNA is uniformly distributed throughout embryonic and extraembryonic tissues of the postimplantation embryo (Fig. 2B). *Axin* mRNA was also detected by RT-PCR in 1-cell through blastocyst stage embryos. Form 1 and 2 mRNAs were both present in all adult tissues examined and in ES cells (Fig. 2C).

4. The predicted amino acid sequences of Axin and its human and chicken homologs

The murine *Axin* (*mAxin*) cDNA sequence included an open reading frame (ORF) beginning at base 3, which could encode a protein of up to 956 (form 1) or 992 (form 2) amino acids (Fig. 3A). Homology searches identified several ESTs representing a human *Axin* homolog, and additional human cDNA sequences were isolated by 5' and 3' RACE (Chenchik et al., 1995). The predicted human and murine Axin amino acid (aa) sequences are 87% identical overall. In addition, a cDNA clone representing a chicken homolog was isolated, and its predicted aa sequence was 66% identical to mouse Axin (Fig. 3A). The first two AUG codons in the *mAxin* ORF were located at bp 375 and 391 of the cDNA, but neither was surrounded by a consensus initiation site (Kozak, 1986). To determine whether either site could serve as an initiation codon, 293T cells were transfected with *Axin* cDNA expression vectors including a C-terminal epitope tag. The sizes of the *in vivo*

translation products were consistent with initiation at one of the first two AUG codons. However, because the murine and human ORFs continue to be conserved upstream of this position, it remains possible that the normal initiation site is further upstream.

The predicted aa sequence contains multiple sites for Ser/Thr phosphorylation and one for Tyr phosphorylation, suggesting that Axin may be a phosphoprotein. It also contains one (form 2) or two (form 1) sequences matching the consensus for a bipartite nuclear localization signal (NLS) (Dingwall and Laskey, 1991). However, detection of epitope-tagged Axin proteins expressed in mammalian cells or Xenopus embryos indicated a peri-membrane rather than a nuclear location for both forms. Database searches revealed two regions of homology to other known proteins. One of these, aa 213 to 338, shows 30-40% identity and 50-60% similarity to the RGS (Regulation of G-protein Signaling) domain (Dohlman and Thorners, 1997). A second potentially important region of similarity (Fig 3c) is a 51 aa segment near the Axin C-terminus, which is ~40% identical and ~60% similar to a conserved sequence near the N-terminus of Drosophila Dsh and its vertebrate homologs (Klingensmith et al., 1994; Sussman et al., 1994). Both the RGS and Dsh homology regions are highly conserved among mouse, human and chick Axin homologs (Fig. 3A).

5. Injection of *Axin* mRNA inhibits dorsal axis formation in Xenopus embryos

The observation that the $Axin^{Tg1}$ and $Axin^{Kb}$ alleles, which cause axial duplications in homozygous mouse embryos, are both unable to produce the major 3.9 kb mRNA suggested that one function of *Axin* is to negatively regulate an early step in axis formation. Because the Axin sequence is highly conserved among amniotes, mAxin might be able to function in amphibian embryos, a system highly amenable to experimental manipulation of early axial development. Therefore, *in vitro* synthesized *mAxin* mRNA (encoding aa 13-956, with an N-

terminal Myc epitope tag) was injected into the dorsal, subequatorial region of 4-cell Xenopus embryos, which were scored at the tadpole stage for effects on axis formation (Fig. 4A and Table 1). Most of these embryos developed with strong axial defects ranging from loss of anterior structures to complete lack of body axis, a phenotype characteristic of completely ventralized embryos (Kao and Elinson, 1988). Control injections of *β-gal* mRNA had no effect. Embryos injected dorsally with *Axin* also showed a markedly reduced expression of the dorsal markers *Siamois*, *Goosecoid*, *Chordin* and *Xnr3*, consistent with the observed ventralizing effects (Fig. 4B). Forms 1 and 2 *Axin* mRNAs were equally active, and α-Myc staining showed that both proteins were similarly localized in a punctate pattern near the plasma membrane.

Table 1. Frequency and extent of ventralization by dorsal injection of *Axin* mRNA, and rescue by *β-catenin* or *Siamois* but not *Xwnt8*.

Both dorsal blastomeres of 4-cell embryos were injected in the subequatorial region. Dorso-Anterior Index (DAI) is a measure of axial development; where 5 is normal, 0 is completely ventralized, and >5 is hyper-dorsalized (Kao and Elinson, 1988). * DAI < 4 was considered significant ventralization.

mRNAs injected	%	Average DAI	Number
β-galactosidase (2 ng, control)	3	4.8	31
Axin (2 ng)	78	1.7	118
Axin + *Xwnt8* (10-20 pg)	90	1.9	67
Axin + *β-catenin* (300 pg)	29	4.3	35
Axin + *Siamois* (50-100 pg)	2	5.1	96

6. Inhibition of dorsal axis formation by Axin is mediated by the Wnt signaling pathway

The ventralizing effect of Axin could be due either to

inhibition of Nieuwkoop Center activity, which requires the activation of the Wnt signaling pathway, or to perturbation of further downstream inductive processes, i.e. establishment of the Spemann organizer or BMP-dependent mesodermal patterning. To test whether Axin might exert its effects via the Wnt pathway, mRNA encoding Xwnt8, β-catenin or Siamois was co-injected with *Axin* mRNA into the dorsal, subequatorial region. *Siamois* is a homeobox gene whose expression is specifically activated by Wnt signaling, and which appears to mediate the effects of the Wnt pathway on axis formation (Carnac et al., 1996; Fagotto et al., 1997; Lemaire et al., 1995). Co-injection of *Siamois* or *β-catenin*, but not *Xwnt8*, overcame the ventralizing effect of *Axin*, rescuing normal axis formation in a large proportion of embryos (Fig. 4A and Table 1) and restoring expression of dorsal markers (Fig. 4C).

As *Xwnt8* or several downstream factors can induce a secondary dorsal axis when injected into the ventral side of the embryo, the ability of *Axin* to inhibit secondary axis formation was examined. Co-expression of Axin completely inhibited the axis-inducing activity of Xwnt8, Xdsh (a Xenopus Dsh homolog) and dominant-negative GSK-3, while it did not affect secondary axis formation by β-catenin or Siamois (Figs. 5A, and B). Thus, injection of *Axin* mRNA can block either normal or secondary dorsal axis formation in Xenopus embryos, apparently by interfering with signaling through the Wnt pathway at a level downstream of Wnt, Dsh and GSK-3, and upstream of β-catenin and Siamois.

7. **Expression of Axin does not affect other downstream pathways involved in axis formation.**

Induction of the Spemann organizer can be also mimicked by Activin, a potent mesodermal inducer, which at high concentrations induces dorsal mesoderm. Axin did not inhibit the induction of *Goosecoid* by Activin in the ventral region of early gastrula embryos (Fig. 5C), and had no effect on the formation of an ectopic blastopore lip or a partial

secondary axis in Activin-injected embryos (Fig. 5A). These results are consistent with the conclusion that Activin acts downstream of, or in parallel to, the Wnt pathway (Carnac et al., 1996; Fagotto et al., 1997; Wylie et al., 1996).

Axial patterning is also regulated further downstream by the antagonistic activity of factors secreted by the organizer (Noggin, Chordin, Follistatin) and the ventral mesoderm (BMPs). For instance, ventral expression of Noggin, a natural inhibitor of BMPs, or a dominant-negative truncated BMP receptor (ΔBMPR) causes formation of an ectopic axis (Hogan, 1996). However, *Axin* failed to block the induction of a secondary axis, or the ectopic expression of the dorsal marker *Goosecoid*, by ventral injection of *Noggin* or *ΔBMPR* (Figs. 5A, C). These results confirm that Axin acts specifically on the Wnt signaling pathway, and does not perturb other pathways involved in early axial patterning.

8. Deletion of the *Axin* RGS homology region creates a dominant-negative mutant.

To test the importance of the RGS domain, we injected Xenopus embryos with mRNA encoding *ΔRGS*, a mutant form of *Axin* in which the sequences encoding aa 252-351 were deleted. Dorsal injection of *ΔRGS* revealed that it had lost the ability to ventralize (only 4/44 embryos ventralized, average Dorso-Anterior Index ~ 5). Surprisingly, *ΔRGS* acted as a potent dorsalizer when injected ventrally, producing secondary axes (usually complete, including the most anterior head structures) in 87% of embryos (Figs. 6A-C). *ΔRGS* induced ectopic expression of several dorsal markers, including *Siamois*, consistent with an activation of the Nieuwkoop Center signaling pathway (Fig. 6D). Ventral injection of wild type *Axin* mRNA had no effect on development and did not induce ectopic expression of dorsal markers (Figs. 6C, and D). However, co-injection of *Axin* blocked the axis-inducing activity of *ΔRGS*, as did co-injection of *C-cadherin*, which binds to and inhibits signaling through β-catenin (Fagotto et al., 1996) (Fig.

6C). Thus, ΔRGS has an effect opposite to that of Axin, and appears to acts through a dominant-negative mechanism to inhibit an endogenous Axin activity. The axial duplications induced by ΔRGS are reminiscent of those caused by loss-of-function *Axin* alleles in the mouse embryo, two examples of which are shown in Figs. 6 (E-G).

Discussion

Mutations at the *Fused* locus have been a subject of interest since the early days of mouse genetics because of their pleiotropic effects on a variety of developmental processes. The most remarkable abnormality seen in early post-implantation embryos homozygous for *Fused* alleles was the formation of ectopic axial structures, which led to the suggestion that this locus played a role in the determination of the embryonic axis (Gluecksohn-Schoenheimer, 1949). The wild type *Fused* gene, now called *Axin*, and a transgenic insertional mutant allele, have been cloned and the structure characterized of $Axin^{Tg1}$. Two of the old mutant alleles, $Axin^{Fu}$ and $Axin^{Kp}$, have also been characterized (Vasicek et al., submitted). The observation that the major *Axin* mRNA is disrupted in two different alleles that cause axial duplications suggested that the normal gene product plays a negative regulatory role at some step in axis formation. This hypothesis is supported by the observation that dorsal injection of *Axin* mRNA blocks axis formation in Xenopus embryos, while ventral injection of a dominant-negative form of *Axin* induces a complete secondary axis. Furthermore, co-injection of *Axin* with factors in the Wnt signal transduction pathway shows that Axin exerts its effects on axis formation by inhibiting the Wnt pathway. These studies reveal that Axin is a novel regulatory protein for a signaling pathway known to trigger an early step in embryonic axis formation in amphibians. Results, together with the phenotype of *Axin* mutant embryos, also imply that the Wnt signaling pathway plays an early and critical role in axis formation in mammalian embryos.

1. The *Axin* gene

The *Axin* gene encodes a major mRNA of 3.9 kb, which is expressed ubiquitously in embryos and adult tissues. In the $Axin^{Tg1}$ allele, exon 2 is replaced with ~600 kb of transgene DNA, preventing expression of the major mRNA. Two spontaneous *Axin* alleles, $Axin^{Fu}$ and $Axin^{Kb}$, are each caused by the insertion of an IAP provirus, within intron 6 or exon 7, respectively (Vasicek et al., submitted). Many of the similarities and differences between the phenotypic effects of *Axin* alleles can be explained by the nature of these mutations. While the provirus in the $Axin^{Fu}$ intron is efficiently spliced out, resulting in near-normal levels of the 3.9 kb mRNA, the provirus in $Axin^{Kb}$ precludes the production of the normal mRNA. Therefore, the similar recessive defects and embryonic lethality seen in $Axin^{Tg1}$ and $Axin^{Kb}$ (but not $Axin^{Fu}$) embryos can be attributed to the inability of either allele to encode the major *Axin* mRNA. On the other hand, the dominant effects of $Axin^{Fu}$ and $Axin^{Kb}$, which are not seen in $Axin^{Tg1}$ mice, appear to be a specific consequence of abnormal transcripts associated with the proviral insertions in these alleles (Vasicek et al., submitted).

Two genomic cosmids encoding part of a human *Axin* homolog map to chromosome 16p13.3 (Accession No. Z69667 and Z81450). Examination of the human genetic map did not reveal any genetic traits (e.g., developmental or neurological defects) that seem likely to be associated with *Axin* mutations.

The predicted Axin protein includes regions of similarity to two families of proteins involved in signal transduction, RGS and Dsh. Several proteins containing an RGS domain (De Vries et al., 1995; Druey et al., 1996; Koelle and Horvitz, 1996) bind G_α subunits (De Vries et al., 1995; Dohlman et al., 1996) and serve as GTPase-activating proteins (GAPs) for the G_i subfamily of G_α subunits, thus inhibiting signal transduction by accelerating the rate of the intrinsic GTPase (Berman et al., 1996; Hunt et al., 1996; Watson et

al., 1996). At least 17 mammalian RGS proteins have been identified, and it is not yet clear if they all serve as GAPs for members of the $G_{i\alpha}$ subfamily, or if some serve as GAPs for other G_{α} subfamilies, or perform other functions (Dohlman and Thorners, 1997). While the Axin RGS domain contains similar residues at many positions of amino acid conservation among RGS proteins, it differs at other conserved positions, and contains two short inserts not present in other RGS domains (Fig. 3B). Thus, whether the Axin RGS is a G_{α} GAP remains to be determined. Axin also displays homology to a 50 aa sequence within a conserved N-terminal region of Drosophila and vertebrate Dsh proteins. The importance of this sequence is unknown, although deletion of a 165 aa segment including this sequence rendered the Drosophila protein inactive (Yanagawa et al., 1995).

2. Axin and Wnt signaling in establishment of the amphibian Nieuwkoop Center

Based on its ability to block ectopic axis formation in Xenopus embryos by Xwnt8, Dsh or dnGSK-3, Axin appears to negatively regulate signaling through the Wnt pathway, either at the level of GSK-3 or further downstream. Furthermore, its inability to block the effects of β-catenin or Siamois suggests that Axin acts upstream of β-catenin (Fig. 7). GSK-3 is a Ser/Thr protein kinase whose activity results in the phosphorylation of β-catenin and its consequent degradation. GSK-3 may directly phosphorylate β-catenin (Yost et al., 1996), or its effects on β-catenin may be mediated by the phosphorylation of adenomatous polyposis coli (APC), which associates with β-catenin and GSK-3 (Miller and Moon, 1996). When GSK-3 activity is inhibited, either naturally through the activity of Wnt and Dsh, or experimentally by dnGSK-3, the level of cytosolic β-catenin is increased, downstream effectors are activated, and Nieuwkoop Center activity results. Thus, both Axin and GSK-3 negatively regulate formation of the Nieuwkoop Center by inhibiting the same signaling pathway.

One hypothetical mechanism for the similar action of Axin and GSK-3 (Fig. 7) is suggested by the observation that Axin can bind to the Ser/Thr protein phosphatase PP2A. If Axin were to inhibit PP2A activity, and if PP2A de-phosphorylated the GSK-3 substrate(s) involved in Wnt signaling, then the overexpression of Axin would increase the level of phosphorylation of this substrate. Thus, even if GSK-3 activity were reduced by Dsh or dnGSK-3, the substrate would remain highly phosphorylated, which could explain why Axin appears epistatic to these two proteins. Alternatively, Axin might stimulate GSK-3 activity by another mechanism that can overcome its inhibition by Dsh or dnGSK-3. A third possibility is suggested by the demonstrated importance of the RGS domain for the ability of Axin to inhibit Wnt signaling and ventralize the frog embryo. There is no evidence for a G_α protein in the Wnt pathway, and the evidence that Axin functions downstream of Dsh and GSK-3 would argue against an activity at the level of a hypothetical G-protein coupled to a Wnt receptor. However, the Axin RGS domain, if it is a functional G_α GAP, might inhibit a second signaling pathway involving a G_α protein, which converges with and stimulates the Wnt pathway at a level downstream of GSK-3. In order to explain the ability of high levels of Axin to block the Wnt pathway, this second signal would have to be *required* for some step in the transmission of the Wnt signal (e.g., cytosolic accumulation of β-catenin) in the early frog embryo.

Not only does deletion of the RGS region abolish the axis-inhibiting properties of Axin, but it creates a dominant-negative form that can induce an ectopic dorsal axis. An obvious implication is that the amphibian embryo contains a protein homologous to Axin, which normally serves to inhibit ectopic axis formation, and whose activity is blocked by ΔRGS. This conclusion is consistent with the observation that loss-of-function *Axin* mutations in the mouse cause the development of ectopic axial structures. Thus, not only is Axin *capable* of inhibiting signaling through the Wnt pathway

when it is over-expressed, but this appears to be a natural function of the protein. Presumably, the levels of endogenous Axin in the embryo are high enough to prevent signaling in the absence of a strong upstream signal (e.g., a Wnt ligand), but low enough to allow signaling when the pathway is activated by a natural or experimental stimulus. While the dominant-negative mechanism of ΔRGS remains to be determined, one possibility is that it competes for binding to a protein to which Axin normally binds, but fails to perform the function carried out by the RGS domain.

3. **Axin, Wnts and axis formation in mammalian embryos**

The ability of Axin to regulate an early step in Xenopus axis formation mediated by the Wnt signaling pathway, together with the occurrence of axial duplications in *Axin* mutant mouse embryos, argues strongly that an evolutionarily conserved mechanism involving the Wnt pathway plays a critical role in embryonic axis formation in mammals as well as amphibians. In Xenopus, signaling through components of the Wnt pathway is thought to confer Nieuwkoop Center activity to a group of dorsal-vegetal blastomeres, which consequently secrete factors that induce formation of the organizer by the adjacent dorsal mesoderm. In the mouse, the equivalent of the organizer is the node, a group of cells at the anterior end of the primitive streak (Beddington, 1994). Fate mapping of the pre-streak embryo indicates that the node derives from epiblast cells at the future posterior pole (Lawson et al., 1991). While the location of the Nieuwkoop Center equivalent (i.e., the cells that induce formation of the organizer) is unknown, the most likely locations are the posterior extraembryonic or embryonic ectoderm proximal to the cells fated to form the node, or the overlying visceral endoderm (Bachvarova, 1996; Conlon and Beddington, 1995).

During normal mouse embryogenesis, the Wnt signaling pathway is activated in a discrete region of the early egg cylinder, by the localized production of a Wnt or another stimulus

that activates downstream components of the Wnt pathway. This localized signal establishes the A-P axis of the embryo, and the responding cells constitute a Nieuwkoop Center equivalent (NCE). According to this model, the ubiquitously expressed Axin serves to attenuate the response to this signal, so that cells in regions of the embryo not exposed to the signal, or exposed to low levels, do not respond, and only a single NCE is formed at the appropriate developmental stage. In mutant embryos lacking Axin, the Wnt pathway could be inappropriately activated and multiple NCE would result. The presence of multiple, discrete axes in mutant embryos could be explained by lateral inhibition mechanism, whereby once a NCE or organizer is formed, it restricts axis formation in adjacent regions of the embryo (Cooke, 1972; Khaner and Eyal-Giladi, 1989; Ziv et al., 1992).

The hypothesis that the Wnt pathway is important for mammalian axis formation is supported by the observation that ubiquitous expression of Cwnt-8C in transgenic embryos causes axial duplications (Pöpperl et al., 1997). Also consistent is the failure of *β-catenin* null mutant mouse embryos to undergo gastrulation (Haegel et al., 1995). No *Wnt* mutants produced so far have affected early events in axis formation, and the expression of Wnts in pre-streak mouse embryos has not been reported (Moon et al., 1997; Parr and McMahon, 1994), leaving open the question of what signal triggers this pathway in the normal embryo. In the pre-streak chick embryo, on the other hand, *Cwnt-8c* is expressed in the posterior marginal zone, a region possibly equivalent to the Nieuwkoop Center (Hume and Dodd, 1993).

While the earliest stage at which axial duplications originate in *Axin* mutant embryos has not been defined, they appear to occur by the stage at which NCE activity would be expected, i.e., the early egg cylinder. Some of earliest $Axin^{Ki/Ki}$ embryos examined (E7.0 - E7.5) contained a bifurcated epiblast with two discrete amniotic cavities or

two primitive streaks (Gluecksohn-Schoenheimer, 1949; Tilghman, 1996). At later stages, in addition to duplication of anterior axial mesoderm (e.g., Figs. 7F, and G), duplication of the allantois (derived from posterior streak) has been observed in both *Axin*^{Tg1} and *Axin*^{fu} embryos (Gluecksohn-Schoenheimer, 1949; Perry et al., 1995). In contrast, transplantation of the node to an ectopic site resulted in formation of ectopic notochord, neural tube and somites, but not allantois (Beddington, 1994). Therefore, structures derived from the most anterior and posterior portions of the primitive streak are duplicated in *Axin* mutant embryos, suggesting that the duplications precede the formation of the streak. The extent of anterior neuroectodermal development of the ectopic axes in *Axin* mutant embryos remains to be further studied using molecular markers: "complete twinning" as well as partial duplications were reported in *Axin*^{Ki} embryos, but ectopic forebrain structures have not yet been documented in *Axin*^{Tg1} or *Axin*^{Kb} embryos.

In addition to their effects on axis formation, *Axin* mutations cause neuroectodermal defects (incomplete closure, malformation or truncation of the head folds), cardiac defects and embryonic lethality in homozygotes. It remains to be determined whether these abnormalities are also due to defective regulation of Wnt signaling pathways. Anterior truncations have been observed in transgenic mouse embryos that ubiquitously expressed Cwnt-8C (R. Beddington, personal communication) and in frog embryos ectopically expressing Xwnt-8 after the mid-blastula transition (Christian and Moon, 1993). Therefore, inappropriate Wnt signaling may also account for the neuroectodermal defects in *Axin* mutant embryos. Another interesting question that can now be addressed is the molecular basis of the dominant defects seen in *Axin*^{fu}, *Axin*^{Kb} and *Axin*^{Ki} heterozygotes, which were attributed to gain-of-function mutations (Greenspan and O'Brien, 1986). Analysis of *Axin*^{fu} and *Axin*^{Kb} suggests that their similar dominant effects may be mediated by C-

terminally truncated Axin proteins that are potentially encoded by abnormally spliced transcripts (Vasicek et al., manuscript submitted). It is possible that these abnormal Axin proteins perturb Wnt signaling pathways involved in brain and skeletal development.

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SECOND SECTION

Wnts are a family of secreted polypeptides which play many functions in development and tumorigenesis (Nusse, 1992; Nusse and Varmus, 1992). The earliest Wnt genes to be identified were shown to have oncogenic effects in the mouse mammary gland. Wnt-1 was first identified as a target for activation by insertion of a retrovirus in mammary tumors ((Nusse, 1992; Nusse and Varmus, 1992) and references therein), and inappropriate expression of Wnt-1 can also cause mammary gland tumorigenesis in transgenic mice (Tsukamoto et al., 1988). Wnts are believed to utilize a signal transduction pathway including the following components: The receptors for Wnts are believed to be members of the frizzled family (Bhanot et al., 1996; Chan et al., 199; Wang et al., 1996; Yang-Synder et al., 1996). The next known component of the pathway is Dishevelled (DSH) (Noordermeer et al., 1994), a cytoplasmic protein that is phosphorylated in response to wingless (Yanagawa et al., 1995). Through an unknown mechanism, Dsh inhibits the activity of glycogen synthase kinase-3 (GSK-3). In the absence of a Wnt signal, GSK-3 activity leads (directly or indirectly) to the phosphorylation and consequent degradation of β-catenin. In the presence of a Wnt signal, GSK-3 is inhibited, increasing the cytosolic level of β-catenin, and promoting its interaction with downstream effectors (Behrens et al., 1996; Funayama et al., 1995; Molenaar et al., 1996; Yost et al., 1996). The product of the APC (adenomatous polyposis coli) gene is also involved in Wnt signaling, apparently forming a complex with GSK-3 and β-catenin (Munemitsu et al., 1995; Papkoff et al., 1996; Polakis, 1997; Rubin et al., 1996; Rubinfeld et al., 19930.

Mutations in at least two components of the Wnt signaling pathway, APC, and β-catenin, have been associated with a variety of cancers, including hereditary colorectal cancer (Familial Adenomatous Polyposis coli, of FAP) and melanoma (Morin et al., 1997; Peifer, 1997; Polakis, 1997; Rubinfeld

et al., 1997). While colon cancer is the primary manifestation of FAP, some patients also develop tumors in other sites, such as other regions of the GI tract, in the thyroid, brain and abdominal cavity (Polakis, 1997). The role of APC in tumorigenesis is thought to be mediated by β-catenin. Normally, APC together with GSK-3 regulates the levels of free cytosolic β-catenin, which remain low. When APC is mutated, degradation of β-catenin is disrupted and levels of β-catenin are greatly increased (Peifer, 1997; Polakis, 1997). In addition, mutations in the β-catenin that interfere with its normal regulation have been found in cases of colon cancer (Morin et al., 1997) and melanoma (Rubinfeld et al., 1997).

Axin has been shown to negatively regulate signaling through the Wnt pathway, based on its ability to block induction of a secondary embryonic axis in frog embryos, when co-injected with mRNAs for Wnt, dishevelled or a dominant-negative mutant form of GSK-3 (Zeng et al., 1997). Axin cannot block axis duplication caused by injection of mRNA for β-catenin, indicating that it functions upstream of β-catenin, but downstream of GSK-3. Therefore, Axin is likely to promote the degradation of β-catenin, either directly or indirectly. Overexpression of Axin in pre-cancerous or tumor cells may be expected to counteract the effects of mutations in APC, β-catenin or other associated proteins, which would otherwise increase the levels of cytosolic β-catenin leading to cellular transformation. Therefore, Axin may be useful clinically to counteract the effects of such mutations. In addition, mutations in the Axin gene itself may result in loss of regulation of β-catenin levels and consequent tumorigenesis, i.e., Axin may be a tumor suppressor gene. Such mutations could either be loss-of-function mutations, or dominant-negative mutations. The ability of one such mutation (a deletion of the RGS domain of Axin) to create a dominant negative mutant form of the protein has been demonstrated (Zeng et al., 1997).

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THIRD SERIES OF EXPERIMENTS

The previous series of experiments concluded that Axin could influence a signal transduction pathway that includes the proteins Wnt (members of the Wnt family of proteins), dishevelled, GSK-3, APC and β-catenin. This conclusion was based on experiments in frog embryos, which showed that Axin mRNA injected into the frog embryo could inhibit the ability of several of these proteins to induce an embryonic axis (Zeng et al., 1997, *Cell* 90, 181-192).

Additional biochemical and genetic data support the involvement of Axin in this signaling pathway. Forms of the Axin protein that can bind directly to APC, GSK-3 and β-catenin were found through a number of assays. Binding of Axin to GSK-3 and to β-catenin was demonstrated by biochemical binding experiments in vitro, as well as by co-immunoprecipitation studies. The former type of experiment shows that highly purified preparations of the proteins (e.g., Axin and GSK-3, or Axin and β-catenin) have the capacity to bind directly to each other. The latter type of experiment shows that, in living mammalian cells, the two proteins are present as part of the same protein complex, so that when one is specifically "pulled out" of a cell lysate, the other protein comes along with it. The binding to APC was shown by both of these methods, as well as by a method known as the yeast two-hybrid system (Fields, S. & Song, 1989, O. *Nature* 340, 245-6).

The ability of Axin to bind to these three proteins supports the contention that Axin is a regulator of signaling through this pathway, and suggests a mechanism by which it may function. Since Axin can bind to both GSK-3 and β-catenin, it could stimulate the ability of GSK-3, a serine-threonine kinase, to phosphorylate β-catenin, which is believed to lead to the degradation of the latter protein. β-catenin that escapes degradation is thought to convey the signal, which in some cells leads to cellular transformation and

cancer. By promoting the degradation of β-catenin, Axin, or other molecules that interact with Axin, could inhibit signaling through β-catenin, and thus inhibit cellular transformation (oncogenic transformation, i.e. from a normal cell to a cancer cell) and tumorigenesis. Thus, Axin could act as a "tumor supressor".

What is claimed is:

1. An isolated nucleic acid which encodes Axin.

2. The isolated nucleic acid of claim 1, wherein the nucleic acid is DNA or RNA

3. The isolated nucleic acid of claim 2, wherein the nucleic acid is cDNA or genomic DNA.

4. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes mutant Axin.

5. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes wildtype Axin.

6. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a mouse wildtype Axin.

7. The isolated nucleic acid of claim 1 comprising a nucleic acid having the sequence designated Seq. I.D. No.: 2.

8. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a mouse wildtype having substantially the same amino acid sequence as the sequence designated Seq. I.D. No.: 1.

9. An isolated nucleic acid which encodes a polypeptide comprising the amino acid sequence of Axin.

10. A vector comprising the isolated nucleic acid of claim 1.

11. The vector of claim 10, further comprising a promoter of RNA transcription operatively linked to the nucleic acid.

12. The vector of claim 10, wherein the promoter comprises a bacterial, yeast, insect or mammalian promoter.

13. The vector of claim 10, further comprising plasmid, cosmid, yeast artificial chromosome (YAC), bacteriophage or eukaryotic viral DNA.

14. The cosmid of claim 13 designated GenBank Accession No. Z69667.

15. The cosmid of claim 13, designated GenBank Accession No. Z81450.

16. A host vector system for the production of a polypeptide which comprises the vector of claim 10 in a suitable host.

17. The host vector system of claim 16, wherein the suitable host is a prokaryotic or eukaryotic cell.

18. The host vector system of claim 17, wherein the prokaryotic cell is a bacterial cell.

19. The host vector system of claim 17, wherein the eukaryotic cell is a yeast, insect, plant or mammalian cell.

20. A method for producing a polypeptide which comprises growing the host vector system of claim 16 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

21. A method of obtaining a polypeptide in purified form which comprises:

(a) introducing the vector of claim 10 into a suitable host cell;

(b) culturing the resulting cell so as to produce the polypeptide;

(c) recovering the polypeptide produced in step (b); and

(d) purifying the polypeptide so recovered.

22. A polypeptide comprising the amino acid sequence of Axin.

23. A purified wildtype Axin.

24. A purified mutant Axin.

25. An oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleotides present within a nucleic acid which encodes wildtype Axin without hybridizing to a nucleic acid which encodes mutant Axin.

26. An oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleotides present within the nucleic acid which encodes mutant Axin without hybridizing to a nucleic acid which encodes wildtype Axin.

27. The oligonucleotide of claim 25 or 26, wherein the nucleic acid is DNA or RNA.

28. A nucleic acid having a sequence complementary to the sequence of the isolated nucleic acid of claim 1.

29. An antisense molecule capable of specifically hybridizing with the isolated nucleic acid of claim 4.

30. A method for determining whether a subject carries a mutation in the axin gene which comprises:

(a) obtaining an appropriate nucleic acid sample from the subject; and

(b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid

which encodes mutant Axin so as to thereby determine whether a subject carries a mutation in the axin gene.

31. The method of claim 30, wherein the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises:

(i) contacting the mRNA with the oligonucleotide of claim 26 under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex;

(ii) isolating the complex so formed; and

(iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

32. The method of claim 30, wherein the determining of step (b) comprises:

(i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid of claim 1 with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid;

(ii) isolating the pieces of nucleic acid; and

(iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

33. The method of claim 30, wherein the determining of step (b) comprises:

(i) sequencing the nucleic acid sample of step (a); and

(ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid of claim 5, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

34. The method of claim 30, wherein the determining of step (b) comprises:

(i) amplifying the nucleic acid present in the sample of step (a); and

(ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

35. The method of claim 31, 32 or 33, wherein the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker.

36. The method the claim 35, wherein the detectable marker is a radioactive isotope, a fluorophor or an enzyme.

37. The method of claim 30, wherein the sample comprises blood, tissues or sera.

38. A method for determining whether a subject has a predisposition for cancer which comprises:

(a) obtaining an appropriate nucleic acid sample from the subject; and

(b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has a predisposition for cancer.

39. The method of claim 38, wherein the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein

the determining of step (b) comprises:

(i) contacting the mRNA with the oligonucleotide of claim 26 under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex;

(ii) isolating the complex so formed; and

(iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

40. The method of claim 38, wherein the determining of step (b) comprises:

(i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid of claim 1 with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid;

(ii) isolating the pieces of nucleic acid; and

(iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

41. The method of claim 38, wherein the determining of step (b) comprises:

(i) sequencing the nucleic acid sample of step (a); and

(ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid of claim 5, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

42. The method of claim 38, wherein the determining of step (b) comprises:

(i) amplifying the nucleic acid present in the sample of step (a); and

(ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

43. The method of claim 39, 40 or 41, wherein the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker.

44. The method the claim 43, wherein the detectable marker is a radioactive isotope, a fluorophor or an enzyme.

45. The method of claim 38, wherein the sample comprises blood, tissues or sera.

46. The method of claim 38, wherein the cancer comprises breast, colorectal, gastrointestinal, esophageal carcinomas or melanomas.

47. A method for treating a subject who has a predisposition to cancer which comprises introducing the isolated nucleic acid of claim 5 into the subject under conditions such that the nucleic acid expresses wildtype Axin, so as to thereby treat the subject.

48. A method for treating a subject who has a predisposition to cancer which comprises an effective amount of the wildtype human homolog of Axin and a pharmaceutically acceptable carrier, so as to thereby treat the subject who is susceptible to cancer.

49. The method of claim 47 or 48, wherein the cancer comprises brain, breast, colorectal, gastrointestinal, esophageal or thyroid carcinomas.

50. A method for determining whether a subject has cancer,

which comprises:

(a) obtaining an appropriate nucleic acid sample from the subject; and

(b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has cancer.

51. The method of claim 50, wherein the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding mutant Axin, and wherein the determining of step (b) comprises:

(i) contacting the mRNA with the oligonucleotide of claim 26 under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex;

(ii) isolating the complex so formed; and

(iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

52. The method of claim 50, wherein the determining of step (b) comprises:

(i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid of claim 1 with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid;

(ii) isolating the pieces of nucleic acid; and

(iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes

mutant Axin.

53. The method of claim 50, wherein the determining of step (b) comprises:

(i) sequencing the nucleic acid sample of step (a); and

(ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid of claim 5, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

54. The method of claim 50, wherein the determining of step (b) comprises:

(i) amplifying the nucleic acid present in the sample of step (a); and

(ii) detecting the presence of mutant Axin in the resulting amplified nucleic acid.

55. The method of claim 51, 52 or 53, wherein the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker.

56. The method the claim 55, wherein the detectable marker is a radioactive isotope, a fluorophor or an enzyme.

57. The method of claim 50, wherein the sample comprises blood, tissue or sera.

58. The method of claim 50, wherein the cancer comprises brain, breast, colorectal, gastrointestinal, esophageal, thyroid carcinomas or melanomas.

59. A method for detecting a mutation in cancerous cells of the subject which comprises:

(a) obtaining an appropriate nucleic acid sample from the subject; and

(b) determining whether the nucleic acid sample from

step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby detect a mutation in the cancerous cells of the subject.

60. The method of claim 59, wherein the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding mutant Axin, and wherein the determining of step (b) comprises:

(i) contacting the mRNA with the oligonucleotide of claim 26 under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex;

(ii) isolating the complex so formed; and

(iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

61. The method of claim 59, wherein the determining of step (b) comprises:

(i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid of claim 1 with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid;

(ii) isolating the pieces of nucleic acid; and

(iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

62. The method of claim 59, wherein the determining of step (b) comprises:

(i) sequencing the nucleic acid sample of step (a); and

(ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid of claim 5, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

63. The method of claim 59, wherein the determining of step (b) comprises:

(i) amplifying the nucleic acid present in the sample of step (a); and

(ii) detecting the presence of mutant Axin in the resulting amplified nucleic acid.

64. The method of claim 61, 62 or 63, wherein the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker.

65. The method the claim 64, wherein the detectable marker is a radioactive isotope, a fluorophor or an enzyme.

66. The method of claim 59, wherein the sample comprises blood, tissue or sera.

67. The method of claim 59, wherein the cancer comprises brain, breast, colorectal, gastrointestinal, esophageal, thryoid carcinomas or melanomas.

68. A method of suppressing cells unable to regulate themselves which comprises introducing the isolated nucleic acid of claim 5 into the cells.

69. The method of claim 68, wherein introducing the isolated nucleic acid, comprises:

(a) recovering cells unable to regulate themselves from the subject;

(b) introducing the isolated nucleic acid of claim 5

into the cells; and

(c) reintroducing the cells of step (b) into the subject so as to suppress those cells.

70. The method of claim 68, wherein the cells are cancerous.

71. The method of claim 70, wherein the cancerous cells are derived from the abdominal cavity, brain, breast, skin, colon, rectum, esophagus, stomach, thryoid or intestine.

72. The method of claim 68, wherein the cells are unable to regulate themselves because of a mutation in the axin gene.

73. The method of claim 68, wherein the cells are unable to regulate themselves of a mutation in the adenomatous polyposis coli gene.

74. A method of suppressing cells unable to regulate themselves which comprises introducing wildtype Axin into the cells in an amount effective enough to suppress the cells.

75. The method of claim 74, wherein the cells are cancerous.

76. The method of claim 75, wherein the cancerous cells are derived from the abdominal cavity, brain, breast, skin, colon, rectum, esophagus, stomach, thyroid or intestine.

77. The method of claim 68, wherein the cells are unable to regulate themselves because of a mutation in the axin gene.

78. The method of claim 68, wherein the cells are unable to

regulate themselves of a mutation in the adenomatous polyposis coli gene.

79. A method of treating a subject who has cancer which comprises introducing the isolated nucleic acid of claim 5, into the subject so as to thereby treat the cancer.

80. The method of claim 79, wherein introducing the isolated nucleic acid, comprises:
(a) recovering cancerous cells from the subject;
(b) introducing the isolated nucleic acid of claim 5 into the cells; and
(c) reintroducing the cells of step (b) into the subject so as to treat the subject who has cancer.

81. The method of claim 80, wherein the cancerous cells are derived from the abdominal cavity, brain, breast, colon, skin, rectum, esophagus, stomach, thyroid or intestine.

82. A method for identifying a chemical compound which is capable of suppressing cells unable to regulate themselves in a subject which comprises:
(a) contacting mutant Axin with the chemical compound under conditions permitting binding between the mutant Axin and the chemical compound;
(b) detecting specific binding of the chemical compound to the mutant Axin; and
(c) determining whether the chemical compound inhibits the mutant Axin so as to identify a chemical compound which is capable of suppressing cells unable to regulate themselves.

83. The method of claim 82, wherein the cells are cancerous.

84. The method of claim 83, wherein the cancerous cells are

derived from the abdominal cavity, brain, breast, skin, colon, rectum, esophagus, stomach, thyroid or intestine..

85. A pharmaceutical composition comprising the chemical compound identified by the method of claim 82 in an amount effective to inhibit cancer and a pharmaceutically effective carrier.

86. A pharmaceutical compositions comprising the antisense molecule of claim 25 in an amount effective to treat cancer and a pharmaceutically effective carrier.

87. A pharmaceutical composition comprising the purified wildtype Axin in an amount effective to treat cancer and a pharmaceutically effective carrier.

88. A method of treating a subject who has cancer comprising administration of an effective amount of the pharmaceutical composition of claim 85, 86 or 87 to the subject who has cancer.

89. The method of claim 88, wherein the administration comprises, topical, oral, aerosol, subcutaneous administration, infusion, intralesional, intramuscular, intraperitoneal, intratumoral, intratracheal, intravenous injection, or liposome-mediate delivery.

90. A transgenic, nonhuman mammal comprising the isolated nucleic acid of claim 1.

91. The transgenic, nonhuman mammal of claim 90, wherein the nucleic acid encodes mutant Axin.

92. The method of claim 47, wherein the nucleic acid is overexpressed so as to thereby treat the subject.

93. A method of inhibiting tumorigenesis in a subject which

comprises administering an effective amount of a molecule which induces degradation of β-catenin thereby inhibiting tumorigensis.

94. The method of claim 94, wherein the molecule is Axin or a functionally active mutant of Axin.

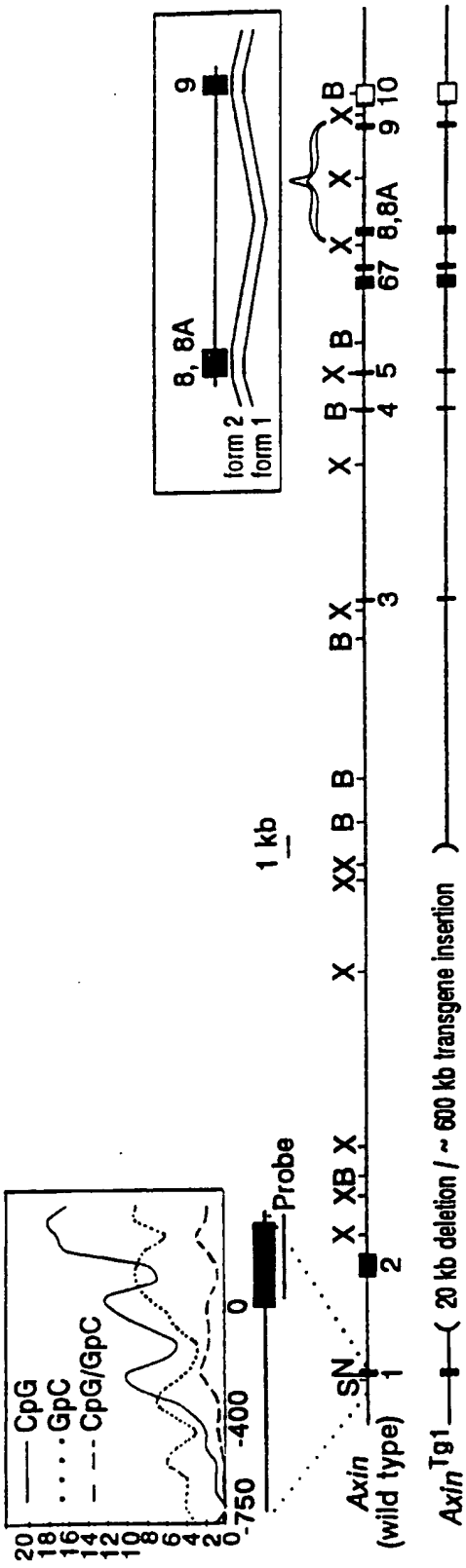
95. The method of claim 94, wherein the molecule is a molecule which inhibits an enzyme.

96. A method of treating a subject with cancer which comprises administering an effective amount of a pharmaceutical composition comprising a molecule which induces degradation of β-catenin and a pharmaceutically acceptable carrier, thereby treating the subject with cancer.

97. The method of claim 96, wherein the molecule which induces degradation of β-catenin is Axin or a functionally active mutant of Axin.

98. The method of claim 96, wherein the molecule is a molecule which inhibits an enzyme.

FIG. 1


CpG
GpC
CpG/GpC
20
18
16
14
12
10
8
6
4
2
0
-750
-400
0
Probe
1 kb
Axin (wild type)
Axin Tg1
20 kb deletion / ~ 600 kb transgene insertion
S
N
X
B
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2
3
4
5
67
8,8A
9
10
form 2
form 1
8, 8A
9

FIG. 2A

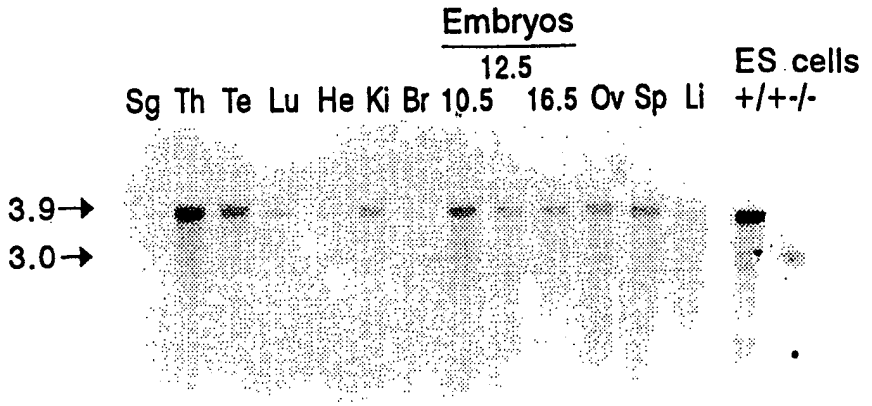


FIG. 2B

FIG. 2C

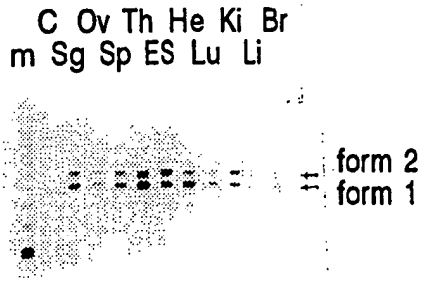
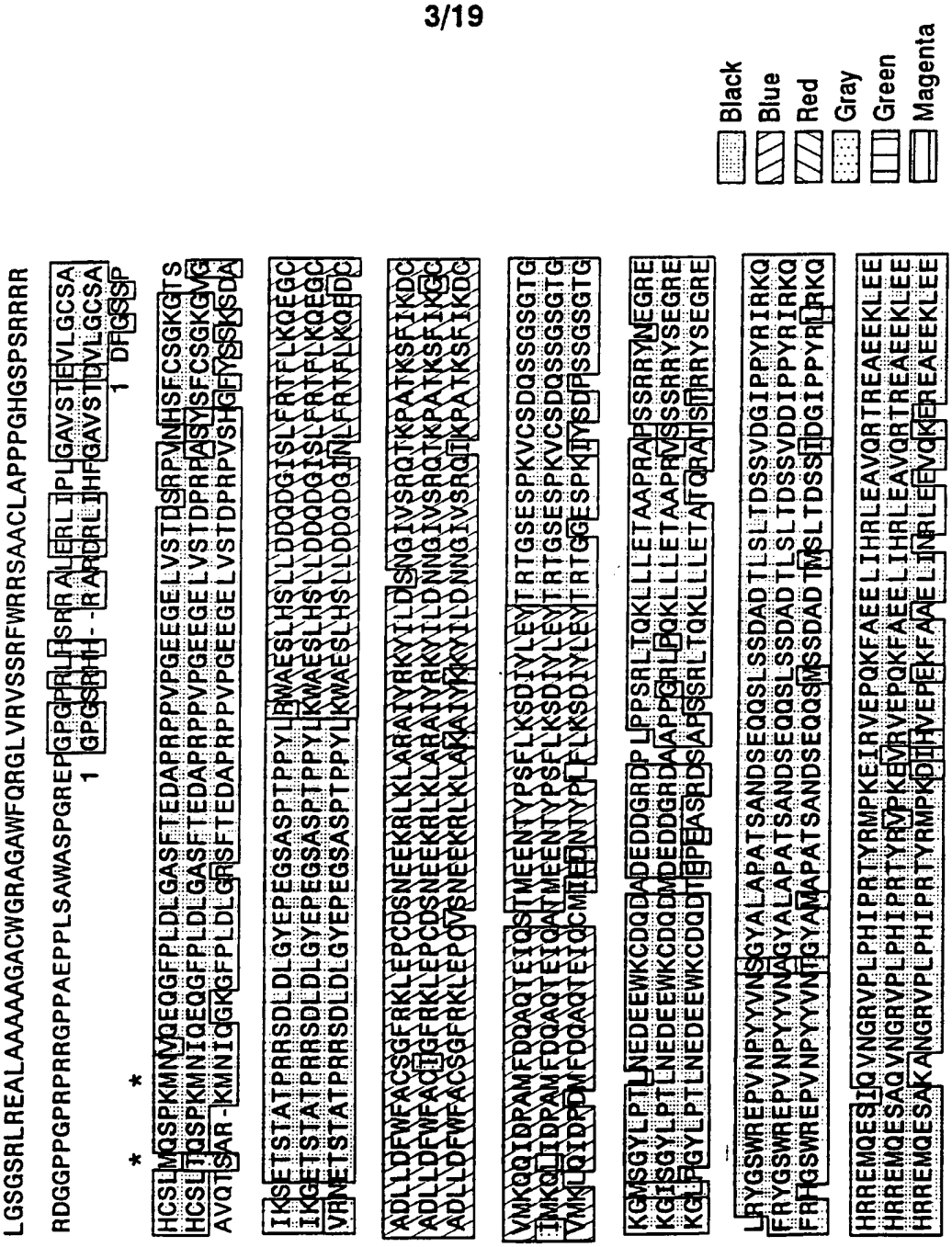


FIG. 3A



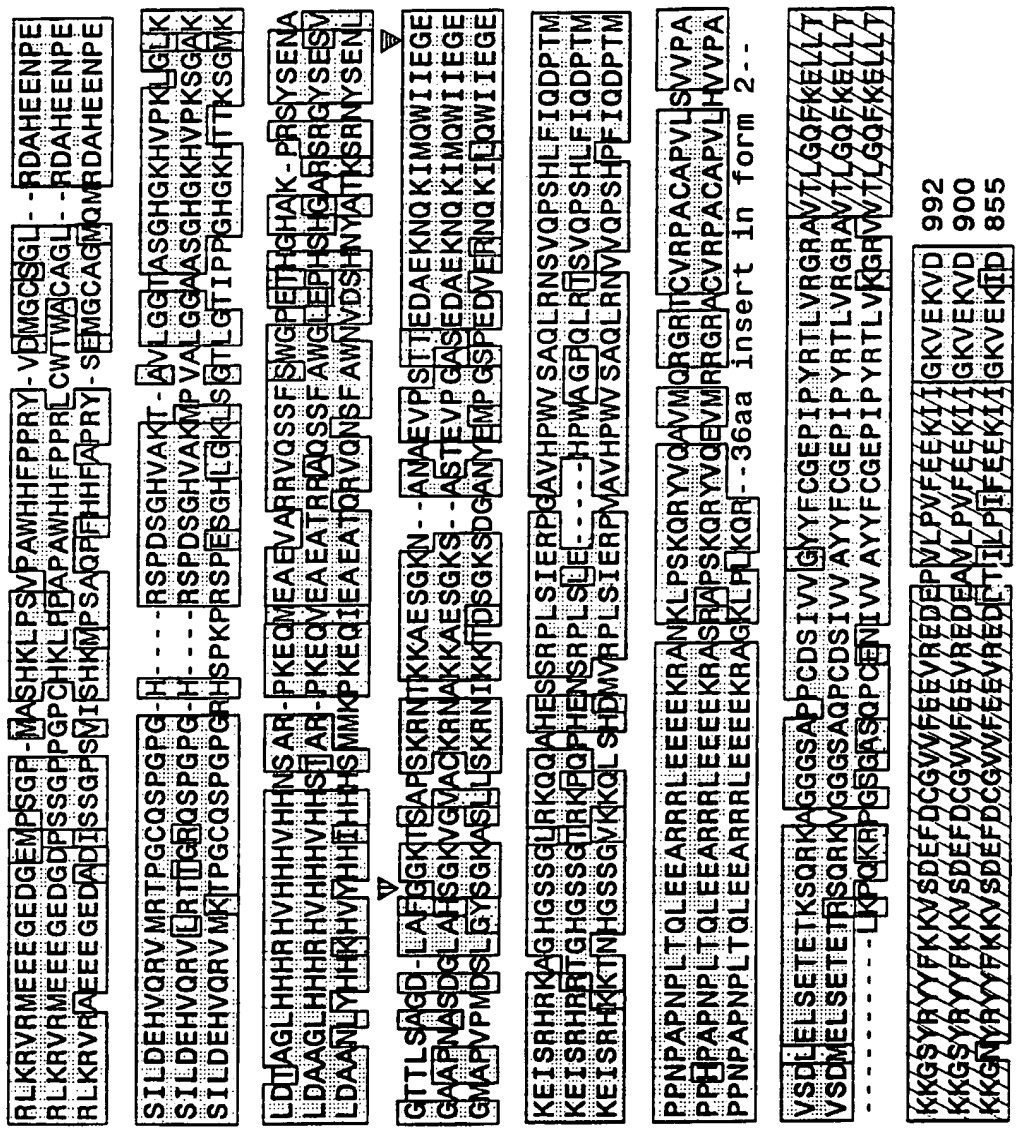


FIG. 3B

FIG. 3C

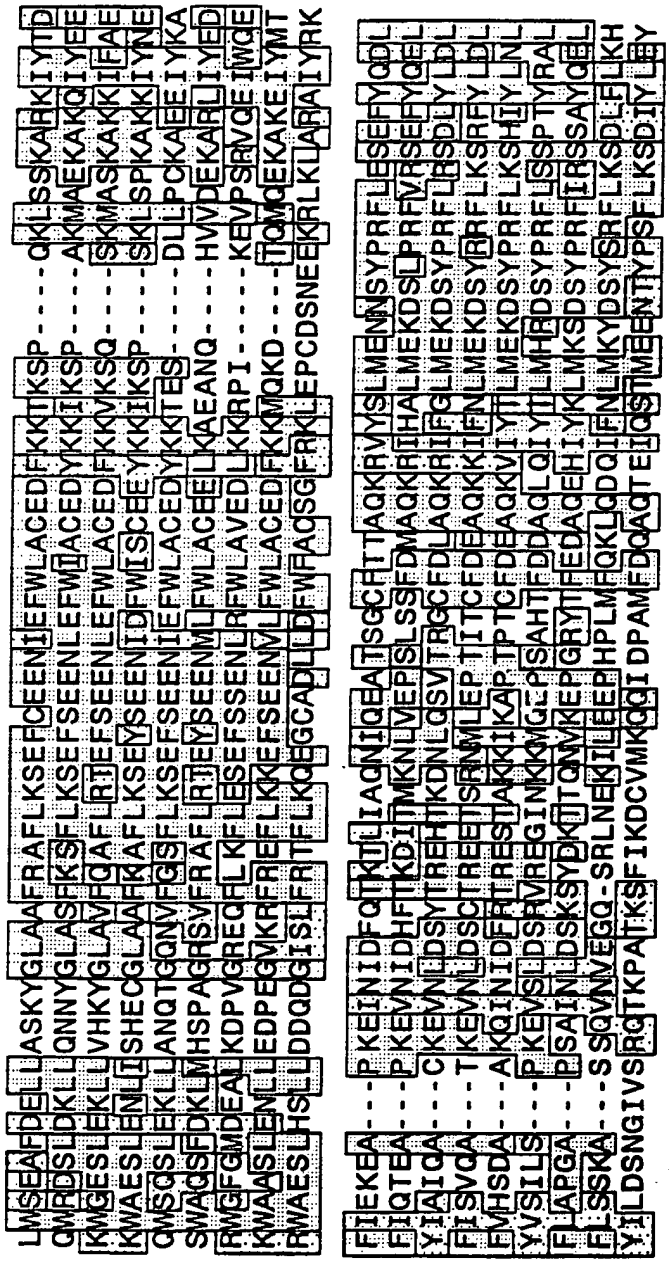


FIG. 3D

FIG. 4A-1

Axin

FIG. 4A-2

Axin + Xwnt8

FIG. 4A-3

Axin + ß-catenin

FIG. 4A-4

Axin + Siamois

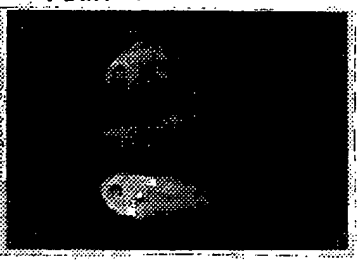


FIG. 4A-5

Control (ß-galactosidase)

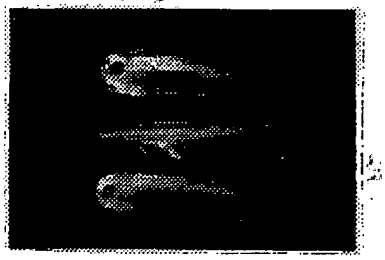


FIG. 4B

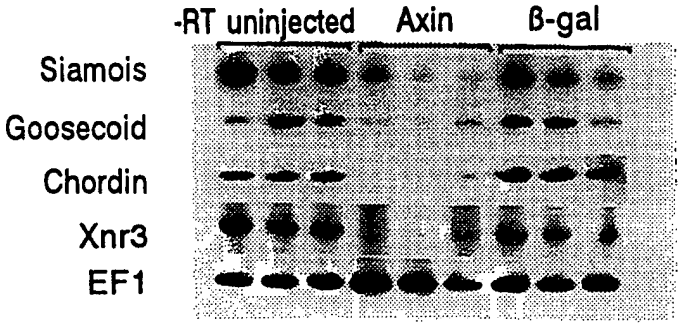

-RT uninjected
Axin
β-gal
Siamois
Goosecoid
Chordin
Xnr3
EF1

FIG. 4C

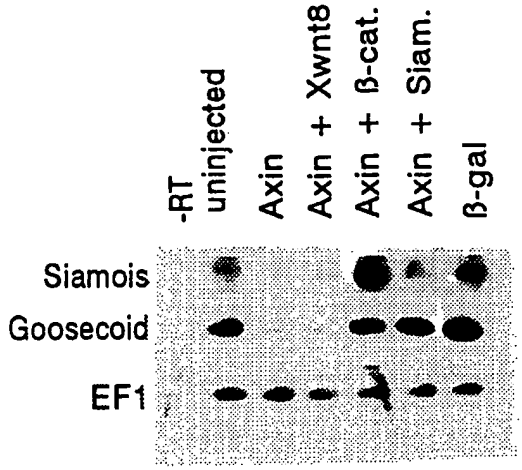

-RT uninjected
Axin
Axin + Xwnt8
Axin + β-cat.
Axin + Siam.
β-gal
Siamois
Goosecoid
EF1

FIG. 5A

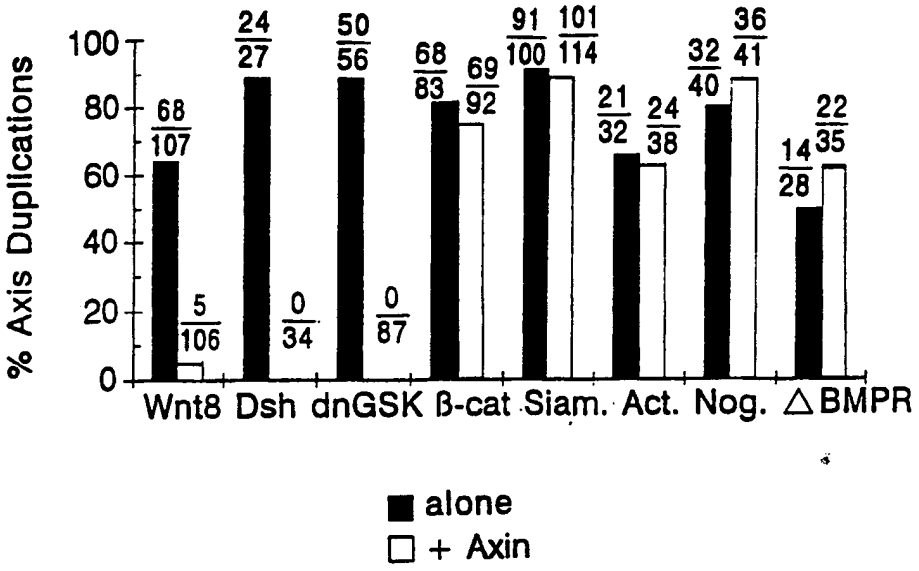

% Axis Duplications
100
80
60
40
20
0
68/107
5/106
24/27
0/34
50/56
0/87
68/83
69/92
91/100
101/114
21/32
24/38
32/40
36/41
14/28
22/35
Wnt8
Dsh
dnGSK
ß-cat
Siam.
Act.
Nog.
△BMPR
alone
+ Axin

FIG. 5B-1 dnGSK-3 dnGSK-3 + Axin FIG. 5B-2

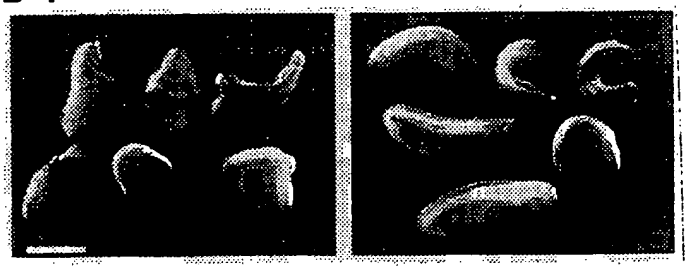


FIG. 5B-3 ß-catenin ß-catenin + Axin FIG. 5B-4

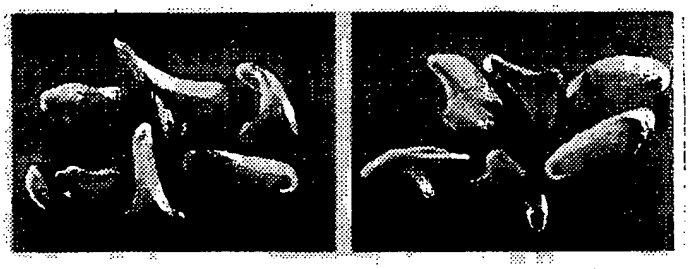


FIG. 5B-5 Siamois Siamois + Axin FIG. 5B-6

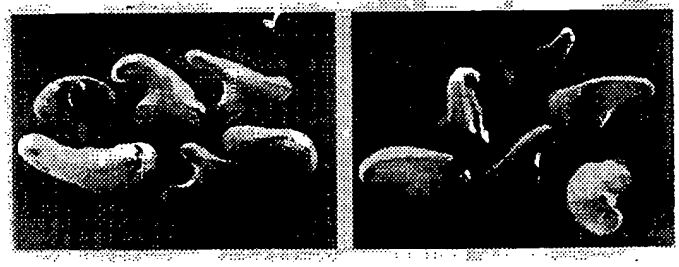


FIG. 5C

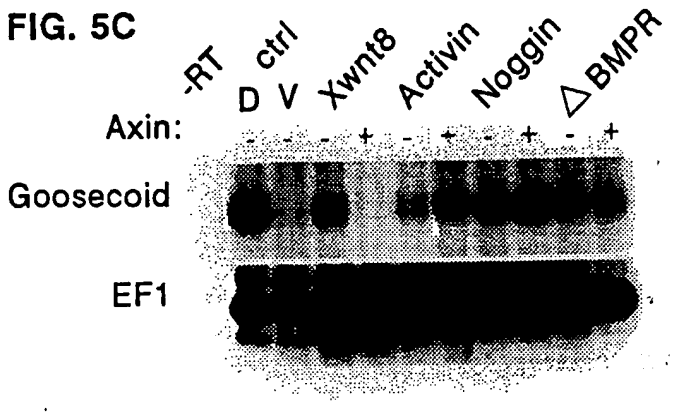


FIG. 6A

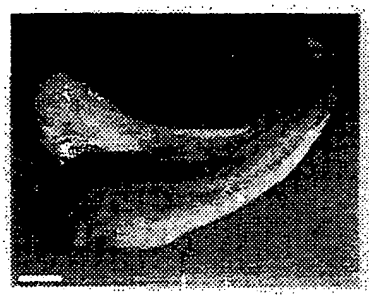
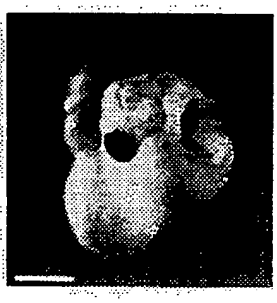


FIG. 6B



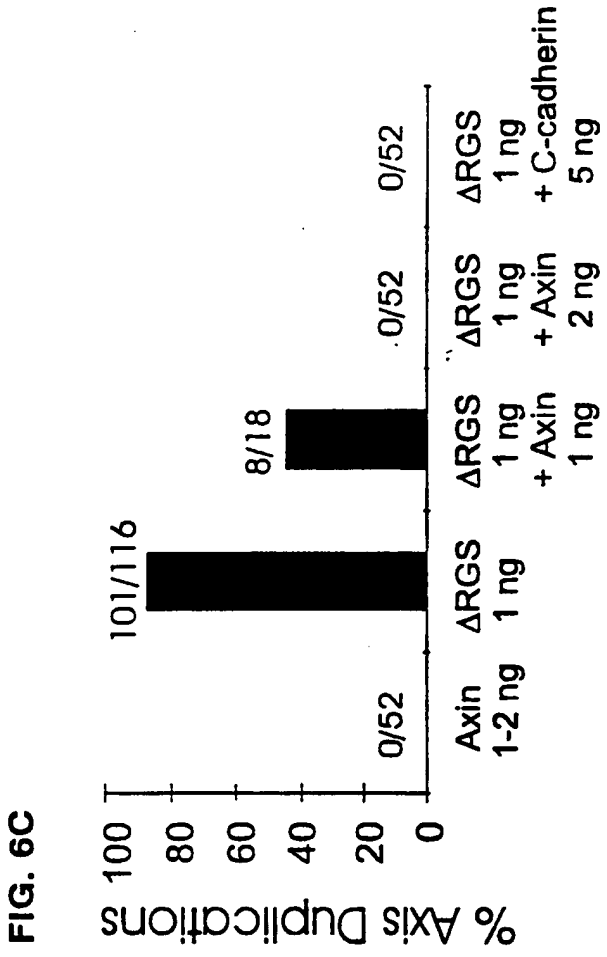

% Axis Duplications
100
80
60
40
20
0
0/52
101/116
8/18
0/52
0/52
Axin 1-2 ng
ΔRGS 1 ng
ΔRGS 1 ng + Axin 1 ng
ΔRGS 1 ng + Axin 2 ng
ΔRGS 1 ng + C-cadherin 5 ng

FIG. 6C

FIG. 6D

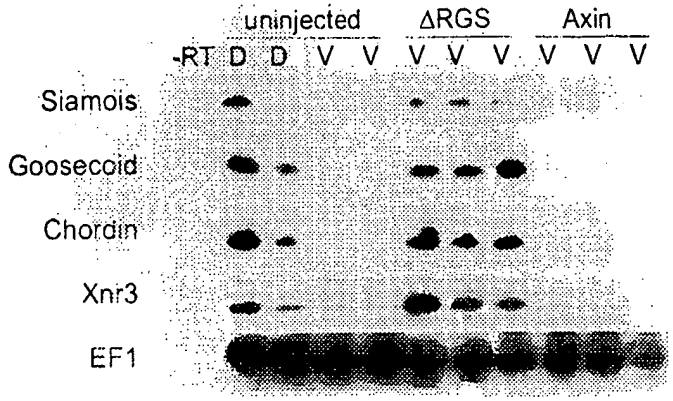


FIG. 6E FIG. 6F FIG. 6G

FIG. 7

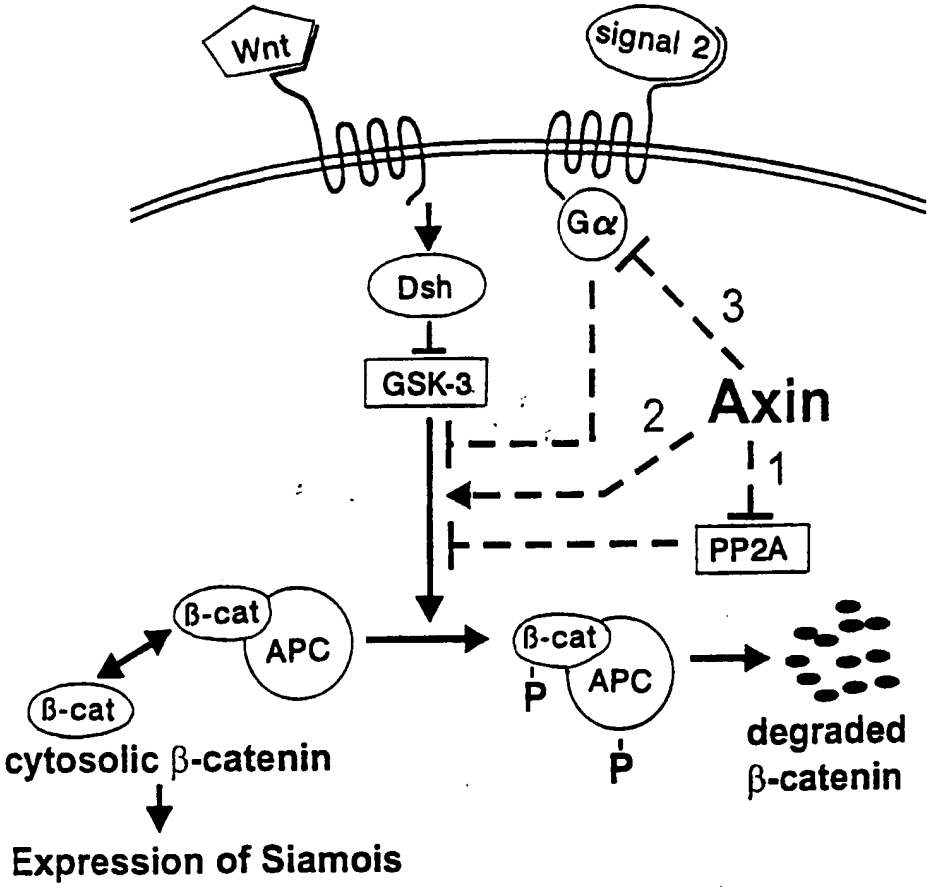

Wnt
signal 2
Gα
Dsh
GSK-3
3
2
Axin
1
PP2A
β-cat
APC
β-cat
cytosolic β-catenin
β-cat
APC
P
P
degraded
β-catenin
Expression of Siamois

FIG. 8

/translation="LGSGSRLREALAAAAAGACWGRAGAWFQRGLVRVSSRFWRRSAA
CLAPPPGHGSPSRRRRRDGGPPGPRPRRGPPAEPPLSAWASPGREPGPGPGPRLHSRRAL
ERLIPLGAVSTEVLGCSAHCSLMQSPKMNVQEQGFPLDLGASFTEDAPRPPVPGEEGE
LVSTDSRPVNHSFCSGKGTSIKSETSTATPRRSDLDLGYEPEGSASPTPPYLRWAESL
HSLLDDQDGISLFRTFLKQEGCADLLDFWFACSGFRKLEPCDSNEEKRLKLARAIYRK
YILDSNGIVSRQTKPATKSFIKDCVMKQQIDPAMFDQAQTEIQSTMEENTYPSFLKSD
IYLEYTRTGSESPKVCSDQSSGSGTGKGMSGYLPTLNEDEEWKCDQDADEDDGRDPLP
PSRLTQKLLLETAAPRAPSSRRYNEGRELRYGSWREPVNPYYVNSGYALAPATSANDS
EQQSLSSDADTLSLTDSSVDGIPPYRIRKQHRREMQESIQVNGRVPLPHIPRTYRMPK
EIRVEPQKFAEELIHRLEAVQRTREAEEKLEERLKRVRMEEEGEDGEMPSGPMASHKL
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GHVAKTAVLGGTASGHGKHVPKLGLKLDTAGLHHHRHVHHHVHHNSARPKEQMEAEVA
RRVQSSFSWGPETHGHAKPRSYSENAGTTLSAGDLPFGGKTSAPSKRNTKKAESGKNA
NAEVPSTTEDAEKNQKIMQWIIEGEKEISRHRKAGHGSSGLRKQQAHESSRPLSIERP
GAVHPWVSAQLRNSVQPSHLFIQDPTMPPNPAPNPLTQLEEARRRLEEEEKRANKLPS
KQRYVQAVMQRGRTCVRPACAPVLSVVPAVSDLELSETETKSQRKAGGGSAPPCDSIV
VGYYFCGEPIPYRTLVRGRAVTLGQFKELLTKKGSYRYYFKKVSDEFDCGVVFEEVRE
DEPVLPVFEEKIIGKVEKVD"

FIG. 9A

```
   1 ctctgggctc aggctcccgg ctcagggaag cgcttgcggc cgccgccgca ggagcctgct
  61 ggggtcgggc tggagcctgg ttccagagag ggctggtgag agtgagcagc cggtttggc
 121 ggcgctctgc ggcctgcctt gcgccgcctc cgggccacgg gtcgccgagc cgccgccgcc
 181 gccgcgatgg gggccccccca gggccgcgcc cccgccgtgg cccgcccgct gagccgccgc
 241 tgagcgcatg ggcctcgccg ggccgggagc caggccccgg gccgcgcctc cacagccgcc
 301 gcgcgctgga gagattgatt cccttgggag ctgtaagtac tgaggtatta gggtgcagcg
 361 ctcattgttc actgatgcag agtcccaaaa tgaatgtcca ggagcagggt ttcccccttgg
 421 acctcggagc aagtttcacc gaagatgccc cccggccccc agtgcctgga gaagagggag
 481 aactggtatc tactgattcg aggcctgtca accacagttt ctgttctggg aaaggtacca
 541 gcattaaaag tgagacctca acagccaccc caagacgttc agatctggat ctgggatatg
 601 agcccgaggg cagtgcctcc cccacccccac catatttgag gtgggctgag tcactgcatt
 661 ccttactgga tgaccaagat gggatcagcc tgttcaggac ttccctgaag caggagggct
 721 gtgctgacct gctggacttc tggtttgcct gcagtggctt caggaagctt gagccctgtg
 781 actcaaatga ggaaaagagg ctgaagctgg caagagccat ctaccgaaag tacatcctgg
 841 atagcaatgg cattgtgtcc agacaaacca agccagccac taagagcttc ataaaggact
 901 gtgtcatgaa gcagcagata gatcctgcca tgtttgacca ggcacagaca gaaatccagt
 961 ccaccatgga ggagaatacc taccttccct ttcttaagtc tgacatttat ttggagtaca
1021 caaggacagg ctcagagagt ccgaaggtct gcagtgacca gagctcaggg tctggaacag
1081 ggaagggcat gtctggatac ctgcccactt tgaatgagga tgaagaatgg aaatgtgacc
1141 aagatgcaga tgaggatgat ggccgagacc ctctccccccc cagcaggctc acccagaagc
1201 tgctattgga gactgctgcc ccgagggccc cctcaagtag acggtacaac gaaggcagag
1261 agctcaggta tggatcttgg agggagcccg tcaaccccta ctacgtcaac tctggctatg
1321 ccctggcccc agccaccagt gccaatgaca gtgagcagca gagcctgtcc agtgatgctg
1381 acacgctatc cttacggac agtagtgtgg atggaatccc cccatacagg atccgtaagc
1441 agcaccgaag ggagatgcag gagagtatcc aagtcaatgg gcgggtacct ctacctcaca
1501 ttcctcgcac ttaccgaatg ccaaaggaga tccgggtaga gccacagaaa ttgctgaag
1561 agcttattca ccgtctagag gctgtccagc gcactcgaga ggctgaagaa aagttggagg
1621 aacggctgaa gcgtgtacgc atggaggaag aaggggagga tggtgaaatg cctctggcc
1681 ccatggcaag tcacaagctg ccttctgtcc cagcttggca ccatttccca ccccgctatg
1741 tggatatggg ctgctctgga ctgcgggatg cccatgagga gaatcctgag agcatcctgg
1801 atgagcacgt gcaaagggtc atgaggacac ctggctgcca gtcacctggc ccaggccacc
1861 gctctccctga cagtgggcat gtggctaaga ctgcagtgct agggggtaca gccctcgggc
```

FIG. 9B

```
1921 atgggaagca tgttcctaag ttagggttga agctggatac agctggcctg caccatcata
1981 gacatgtcca ccaccatgtt caccataatt cagctagacc taaggagcaa atggaggctg
2041 aagttgcccg cagggtccag agcagcttct cgtggggccc agaaacacat ggtcatgcca
2101 agcccggag ctattccgag aacgcaggca ccaccctcag tgctggggat ttgcccttg
2161 gtggtaaaac tagtgcacct tccaaagaa acaccaagaa ggctgaatct gggaagaatg
2221 ccaatgctga ggtacccagt accacagagg acgctgagaa gaaccagaag atcatgcagt
2281 ggatcattga gggagaaag gagatcagta gacaccggaa ggcaggccat gggtcttctg
2341 ggttgaggaa gcagcaggcc catgaaagct ccaggcccct gtccatcgag cgtcctgggg
2401 ccgtgcaccc ctgggtcagc gctcagcttc ggaattctgt ccagccttct catctttca
2461 tccaagatcc cacaatgcca cccaatccag cctaatccc cctgacccag ctggaagagg
2521 cccgcaggcg ttggaagaaa gaagaaaaga gagcaaacaa actgccctcc aagcagaggt
2581 atgtgcaggc agtcatgcag cggggacgca cctgtgtcag gccagcttgt gcaccggtgc
2641 tgagtgtggt accagccgtg tcggacttgg aactctccga gacagagaca aaatcacaaa
2701 gaaaggcagg tggcgggagt gcaccaccat gtgacagcat tgttgtgggc tactatttct
2761 gtggggaacc catcccctac cggaccctgg tgagggccg tgctgtcacc ctgggccagt
2821 tcaaggagct gctaaccaag aaggggagct acagatacta ctttaagaaa gtgagtgatg
2881 agtttgactg tggtgtggta ttgaggaagg tacgggagga tgagcccgtc ttgcctgtct
2941 ttgaagaaaa gatcatcggc aaggtggaaa aggtggactg agcactgggc agcacacccg
3001 gagcacacca tcactactgt gcattgtcat cagtcaggtg gacagcctg tcctcaggag
3061 cctggtgtgg ggaacaacac aagattgtgt catgagctct tctatcgggg tgaggctggg
3121 gacctaggt gtctgccagc ctctgtccct ctggctttgg gaaagtgggg gtgggggtc
3181 gtcctactga gtggttcctt gggttctctt gtttccctgt tcaagaggaa agttccactt
3241 accaccacat taccccctga agcaatacca ggagccatct catgaccctc agcagctctt
3301 gcttctgaat ccagtctgac ctagggatac tttgccctgg gcttgtatcc cactgtcctc
3361 ttctctctct ctgggaccta tccactgcac ctggttgggc tcaggtccag gagcagggga
3421 tcctgtgggg gcctctatat attgtacatg tcactgagtg ccttcaacat agctgtctct
3481 tgcctgccac tgtgtgaatc tggcagctga gtatctcagg ccccttgcc tgtctccagc
3541 caccagcttg gttcagcagg agggggggcg gtgtgtctgg tcccttccaa gtgtccgtgt
3601 aaatatgtac atttctcagg ccagggccag cagggggata ccctgagccc atttttcatg
3661 caatgacttg tacaattatc tttcaaaggg tacttggata ataatgaaat aaaaacgttt
3721 ttgaacctc caaaaaaaa aaaaaaaaaa aaaaaaaaaa a
```

FIG. 10A

```
   1 gggcccgggg tcccgccacc accgcgcgcg ggacagattg attcactttg gagctgtaag
  61 tactgatgta ttagggtgca gcgctcattg ttcattgacg cagagtcccc aaatgaatat
 121 ccaagagcag ggtttcccct tggacctcgg agcaagttcc accgaagatg ctcccgacc
 181 cccagtgcct ggtgaggagg gagaactggt gtccacagac ccgaggcccg ccagctacag
 241 tttctgctcc gggaaaggtg ttggcattaa aggtgagact tcgacggcca ctccgaggcg
 301 ctcggatctg gacctgaggt atgagcctga gggcagtgcc tcccccaccc caccatactt
 361 gaagtgggct gagtcactgc attcccctgct ggatgaccaa gatggataa gcctgttcag
 421 gacttccctg aagcaggagg gctgtgccga ctgctggtac ttctggttg cctgcactgg
 481 cttcaggaag ctggagccct gtgactcgaa cgaggagaag aggctgaagc tggcgagagc
 541 catctaccga aagtacattc ttgataacaa tggcatcgtg tcccggcaga ccaagccagc
 601 caccaagagc ttcataaagg gctgcatcat gaagcagctg atcgatcctg ccatgtttga
 661 ccaggcccag accgaatcc aggccactat ggaggaaaac acctatccct cctccttaa
 721 gtctgatatt tatttggaat atacgaggac aggctcggag agccccaaag tctgtagtga
 781 ccagagctct gggtcaggga cagggaaggg catatctgga tacctgccga cctaaatga
 841 agatgaggaa tggaagtgtg accaggacat ggacgaggac gatggcagag acgctgctcc
 901 ccccggaaga ctccctccag agctgctcct ggagacagct gccccgaggg tctcctccag
 961 tagacggtac agcgaaggca gagagttcag gtatggatcc tggcgggagc cagtcaaccc
1021 ctatatgtc aatgccggct atgccctggc cccagccacc agtgccaacg acagcgagca
1081 gcagagcctg tccagcgatg cagacaccct gtccctcacg gacagcagcg tggatgggat
1141 cccccatac aggatccgta agcagcaccg cagggagatg caggagagcg cgcaggtcaa
1201 tgggcgggtg cccctacctc acattccccg cacgtaccgg gtgccgaagg aggtccgcgt
1261 ggagcctcag aagttcgcgg aggagctcat ccaccgccctg gaggctgtgc agcgcacgcg
1321 ggaggccgag gagaagctgg aggagcggct gaagcgcgtg cgcatggagg aggaaggtga
1381 ggacggcgat ccatcgtcag ggccccccagg gccgtgtcac aagctgcctc ccgccccgc
1441 ttggcaccac ttcccgcccc gcttgtgttg gacatgggct tgtgccgggc tccgggatgc
1501 acacgaggag aaccctgaga gcatcctgga cgagcacgta cagcgtgtgc tgaggacaac
1561 tggccgccag tcgcctgggc ctggccatcg ctccccggac agtgggcacg tggccaagat
```

FIG. 10B

```
1621 gccagtggca ctgggggtg ccgcctcggg gcacgggaag cacgtaccca agtcaggggc
1681 gaagctggac gcggccggcc tgcaccacca ccgacacgtc caccaccacg tccaccacag
1741 cacagcccgg cccaaggagc aggtggaggc cgaggccacc cgcagggccc agagcagctt
1801 cgcctgggggc ctggaaccac acagccatgg ggcaaggtcc cgaggctact cagagagtgt
1861 tggcgctgcc cccaacgcca gtgatggcct cgcccacagt gggaaggtgg gcgttgcgtg
1921 caaaagaaat gccaagaagg ctgagtcggg gaagagcgcc agcaccgagg tgccaggtgc
1981 ctcggaggat gcggagaaga accagaaaat catgcagtgg atcattgagg gggaaaagga
2041 gatcagcagg caccgcagga ccggccacgg gtcttcgggg acgaggaagc cacagcccca
2101 tgagaactcc agaccyttgt cccttgagca ccccttgggcc ggcccttcagc tccggaccctc
2161 cgtgcagccc tcccacctct tcatccaaga ccccaccatg ccacccccacc cagctcccaa
2221 cccccctaacc cagctggagg aggcgcgccg acgtctggag gaggaagaaa ayagagccag
2281 ccgagcaccc tccaagcaga ggtatgtgca ggaggttetg cggcggggac gcgcctgcgt
2341 caggccagcg tgcgcgccgg tgctgcacgt ggtaccagcc gtgtcggaca tggagctctc
2401 cgagacagag acaagatcgc agaggaaggt gggcggcggg agtgcccagc cgtgtgacag
2461 catcgttgtg gcgtactact tctgcgggga acccatcccc taccgcaccc tggtgagggg
2521 ccgcgctgtc accctgggcc agttcaagga gctgctgacc aaaaagggca gctacagata
2581 ctacttcaag aaagtgagcg acgagttga ctgtgggggtg gtgttgagg aggttcgaga
2641 ggacgaggcc gtcctgcccg tctttgagga gaagatcatc ggcaaagtgg agaaggtgga
2701 ctgataggct ggtgggctgg ccgctgtgcc aggcgaggcc cttggcgggc acgggtgtca
2761 cggccaggca gatgacctcg tactcaggag cccgatgggg aacagtgttg ggtgtaccac
2821 ccatccctgt ggtctacccg tgtctagagg caggtagggg gtcccttccaa gtggttccaca
2881 agcttctgtc ctgccccaa ggaggcagcc tggaccactc ctcatagcaa tacttggagg
2941 gcccagccca agtgaggcag ccgaggtccc tyctgccagc ttcaggtgac ccccccccat
3001 cccccggcac ctcccttggg cacgtgtgct gggatctact ttcccctctgg gatttgccca
3061 cgtacccagg tctggctggg gcccaggccc ggatgcagag gcctgcaggg cctctgtcaa
3121 ttgtacgcgc caccaagtgc cttcaacaca gcttgtctct tgcctgccac tgtgtgaatc
3181 ggcgacggag cactgcacct gcctccagcc gccggctgtg cagtcctggg tcctcctttc
3241 tgagggcccg tgtaaatatg tacatrtctc aggctagggc cagcaggggc tgcccgagtc
3301 tgtttttcat gcgatgacac ttgtacaatt atctttcaa aggtacttgg ataataatga
3361 aataaaactg ttttgaacc tgaataaaaa aaaaaaaaaa aaaaaaaaaa a
```

FIG. 11

/translation="GPGSRHHRARDRLIHFGAVSTDVLGCSAHCSLTQSPKMNIQEQG
FPLDLGASFTEDAPRPPVPGEEGELVSTDPRPASYSFCSGKGVGIKGETSTATPRRSD
LDLGYEPEGSASPTPPYLKMAESLHSLLDDQDGISLFRTFLKQEGCADLLDFWFACTG
FRKLEPCDSNEEKRLKLARAIYRKYILDNNGIVSRQTKPATKSFIKGCIMKQLIDPAM
FDQAQTEIQATMEENTYPSFLKSDIYLEYTRTGSESPKVCSDQSSGSGTGKGISGYLP
TLNEDEEWKCDQDMDEDDGRDAAPPGRLPQKLLLETAAPRVSSSRRYSEGREFRYGSW
REPVNPYYVNAGYALAPATSANDSEQQSLSSDADTLSLTDSSVDGIPPYRIRKQHRRE
MQESAQVNGRVPLPHIPRTYRVPKEVRVEPQKFAEELIHRLEAVQRTREAEEKLEERL
KRVRMEEEGEDGDPSSGPPGPCHKLPPAPAWHHFPPRLCWTWACAGLRDAHEENFESI
LDEHVQRVLRTTGRQSPGPGHRSPDSGHVAKMPVALGGAASGHGKHVPKSGAKLDAAG
LHHHRHVHHHVHHSTARPKEQVEAEATRRAQSSFAWGLEPHSHGARSRGYSESVGAAP
NASDGLAHSGKVGVACKRNAKKAESGKSASTEVPGASEDAEKNQKIMQWIIEGEKEIS
RHRRTGHGSSGTRKPQPHENSRPLSLEHPWAGPQLRTSVQPSHLFIQDPTMPPHPAPN
PLTQLEEARRRLEEEEKRASRAPSKQRYVQEVMRRGPACVRPACAPVLHVVPAVSDME
LSETETRSQRKVGGGSAQPCDSIVVAYYFCGEPIPYRTLVRGRAVTLGQFKELLTKKG
SYRYYFKKVSDEFDCGVVFEEVREDEAVLPVFEEKTIGKVEKVD"

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14414

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 320.1, 325; 514/2, 44; 536/23.1, 23.5, 24.5, 24.31; 530/350; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P --- Y,P	Zeng, L. et al., The Mouse Fused Locus Encodes Axin, an Inhibitor of the Wnt Signaling Pathway That Regulates Embryonic Axis Formation. Cell. 11 July 1997,Vol. 90. pages 181-192, especially page 184, Figure 3, page 186, "The Axin Gene," 1st and 2nd paragraphs and page 190, "Experimental Procedures," entire section.	1-15 --- 16-84, 90-92
Y	Fujimoto, J. et al. Progestins and Danazol Effect on Cell-to-cell Adhesion, and E-Cadherin and alpha- and beta- Catenin mRNA Expressions. J. Steroid Biochem. Molec Biol. 1996Vol. 57. No. 5-6. pages 275-282, especially page 278, Figure 6.	93, 96

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 29 SEPTEMBER 1998	Date of mailing of the international search report 26 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PETER TUNG Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14414

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/17, 48/00; C07H 21/04; C07K 14/435; C12N 15/00, 15/63, 15/85; C12Q 1/68

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 69.1, 320.1, 325; 514/2, 44; 536/23.1, 23.5, 24.5, 24.31; 530/350; 800/2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, CANCERLIT, CAPLUS, DISSABS, BIOSIS, EMBASE, WPIDS
SEARCH TERMS: Axin, FU gene, Fused, beta catenin, mouse, locus, therapy, Frank Constantini, Frank Costantini, Li Zheng